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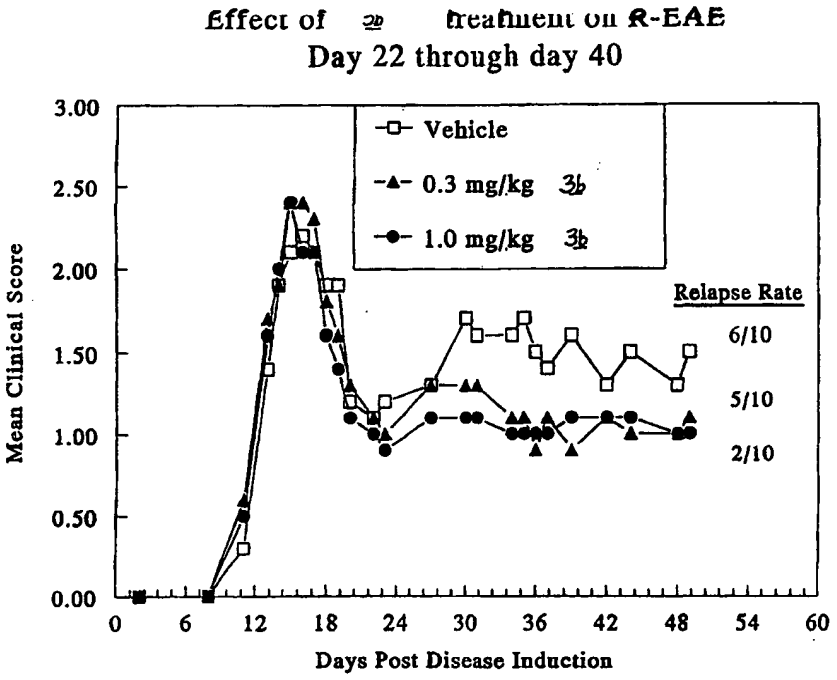
(57) Abstract

This invention is directed to the treatment of inflammatory and autoimmune diseases by administering proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, or mixtures thereof. The invention is further directed to the treatment of inflammatory and autoimmune diseases by administering an effective combination of a glucocorticoid and a proteasome inhibitor, ubiquitin pathway inhibitor, agent that interferes with the activation of NF-κB via the ubiquitin proteasome pathway, or mixture thereof. Pharmaceutical compositions comprising a combination of a glucocorticoid and a proteasome inhibitor, ubiquitin pathway inhibitor, agent that interferes with the activation of NF-κB via the ubiquitin proteasome pathway, or mixture thereof are also contemplated within the scope of the invention.



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PROTEASOME INHIBITORS, UBIQUITIN PATHWAY INHIBITORS OR AGENTS THAT INTERFERE WITH THE ACTIVATION OF NF-κB VIA THE UBIQUITIN PROTEASOME PATHWAY TO TREAT INFLAMMATORY AND AUTOIMMUNE DISEASES

BACKGROUND OF THE INVENTION

Field of the invention

This invention is directed to compositions and methods for treatment of inflammatory and autoimmune diseases.

Summary of the related art

Eukaryotic cells contain multiple proteolytic systems, including lysosomal proteases, calpains, ATP-ubiquitin-proteasome dependent pathway, and an ATP-independent nonlysosomal process. The major neutral proteolytic activity in the cytosol and nucleus is the proteasome, a 20S (700 kDa) particle with multiple peptidase activities. The 20S complex is the proteolytic core of a 26S (1500 kDa) complex that degrades or processes ubiquitin-conjugated proteins. Ubiquitination marks a protein for hydrolysis by the 26S proteasome complex. Many abnormal or short-lived normal polypeptides are degraded by the ubiquitin-proteasome-dependent pathway. Abnormal peptides include oxidant-damaged proteins (e.g., those having oxidized disulfide bonds), products of premature translational termination (e.g., those having exposed hydrophobic groups which are recognized by the proteasome, and stress-induced denatured or damaged proteins (where stress is induced by, e.g., changes in pH or temperature, or exposure to metals). The proteasome also participates in the rapid elimination and post-translational processing of proteins involved in cellular regulation (e.g., cell cycle, gene transcription,

and metabolic pathways), intercellular communication, and the immune response (e.g., antigen presentation).

The transcription factor NF-κB is a member of the Rel protein family. The Rel family of transcriptional activator proteins can be divided into two groups. The first group requires proteolytic processing, and includes p105 and p100, which are processed to p50 and p52, respectively. The second group does not require proteolytic processing and includes p65 (Rel A), Rel (c-Rel), and Rel B. NF-κB comprises two subunits, p50 and an additional member of the Rel gene family, e.g., p 65. Unprocessed p105 can also associate with p65 and other members of the Rel family. In most cells, the p50-p65 heterodimer is present in an inactive form in the cytoplasm, bound to IκB-α. The ternary complex can be activated by the dissociation and destruction of IκB-α, while the p65/p105 heterodimer can be activated by processing of p105.

The ubiquitin-proteasome pathway plays an essential role in the regulation of NF-κB activity, being responsible both for processing of p105 to p50 and for the degradation of the inhibitor protein IκB-α. In order to be targeted for degradation by the proteasome, IκB-α must first undergo selective phosphorylation at serine residues 32 and 36, followed by ubiquitination (Chen *et al. Genes & Development* (1995) *9*:1586; Chen *et al. Cell* (1996) *84*:853; Brockman *et al. Mol. Cell. Biol.* (1995) *15*:2809; Brown *et al. Science* (1995) *267*:1485).

Once activated, NF-κB translocates to the nucleus, where it plays a central role in the regulation of a remarkably diverse set of genes involved in the immune and inflammatory responses (Grilli *et al., International Review of Cytology* (1993) *143*:1-62). For example, NF-κB is required for the expression of a number of genes involved in the inflammatory response, such as TNF-α gene and genes encoding the cell adhesion molecules E-selectin, P-selectin, ICAM, and VCAM (Collins, T., *Lab. Invest.* (1993)

68:499. NF-κB is also required for the expression of a large number of cytokine genes such as IL-2, IL-6, granulocyte colony stimulating factor, and IFN-β. Inducible nitric oxide synthetase is also under regulatory control of NF-κB. Proteasome inhibitors block IκB-α degradation and activation of NF-κB (Palombella *et al.* WO 95/25533 published 9/28/95; Traenckner, *et al.*, *EMBO J.* (1994) 13:5433). Proteasome inhibitors also block TNF-α induced expression of the leukocyte adhesion molecules E-selectin, VCAM-1, and ICAM-1 (Read, *et al.*, *Immunity* (1995) 2:493).

Cyclins are proteins involved in cell cycle control. The proteasome participates in the degradation of cyclins. Cyclin degradation enables a cell to exit one cell cycle stage (e.g., mitosis) and enter another (e.g., division). There is evidence that cyclin is converted to a form vulnerable to a ubiquitin ligase or that a cyclin-specific ligase is activated during mitosis (Ciechanover *Cell* (1994) *79*:13). Inhibition of the proteasome inhibits cyclin degradation, and therefore inhibits cell proliferation (Kumatori *et al. Proc. Natl. Acad. Sci. USA* (1990) *87*:7071)

The continual turnover of cellular proteins by the ubiquitin-proteasome pathway is also used by the immune system to screen for the presence of abnormal intracellular proteins (Goldberg and Rock *Nature* (1993) *357*:375). In this process, lymphocytes continually monitor small fragments of cell protein that are presented on class I major histocompatibility complex (MHC) molecules. Proteasomes initially degrade proteins to small peptides, most of which are rapidly hydrolyzed to amino acids by cytosolic exopeptidases. But some of these peptides are transported into the endoplasmic reticulum where they bind to MHC molecules and are then transported to the cell surface in a process known as antigen presentation. If the peptides are abnormal (for example, if they are derived from viral proteins), they elicit cell destruction by cytotoxic T cells. Inhibitors that prevent proteasome function have been shown to block the generation of most of the peptides presented on MHC class I molecules (Rock, *et al. Cell* (1994) *78*:

761).

Multiple sclerosis (MS) is an incurable neurological illness that frequently causes chronic disability. MS is the most common demyelinating disease of the human central nervous system and typically affects youth and women more than men. Clinically, the illness is characterized into a relapsing-remitting or chronic progressive stage, although more precisely defined stages exist for research purposes. It tends to follow a highly unpredictable course leading to chronic and sometimes devastating disability. It is widely believed that MS is the result of an autoimmune disorder in a genetically susceptible individual, mediated by autoreactive T cells that migrate into the CNS and initiate the inflammatory demyelinating lesion.

Airway hyperreactivity to a variety of spasmogens and pulmonary inflammation characterized by eosinophilia are pathologies that are characteristic of asthma (Beasely, *et al. Am. Rev. Resp. Dis.* (1989) *139*:806). Asthma is a chronic condition of the airways that involves many types of inflammatory cell and the release of many mediators and neurotransmitters that have multiple effects on the various target cells in the airway. The degree and extent of inflammation in the airway wall are broadly related to the clinical severity of the asthma. The inflammatory response of asthma consists of activation of mast cells resident in the airways, increased numbers of lymphocytes (which are mainly $CD4^+$ T lymphocytes) and an infiltration with eosinophils, which appear to degranulate.

There is a need in the art for effective therapies for the treatment of multiple sclerosis or asthma.

BRIEF SUMMARY OF THE INVENTION

The present invention is directed to methods for treating a patient afflicted with multiple sclerosis or asthma comprising administering to said patient an effective amount of an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof.

In certain embodiments of the invention, the agent is a proteasome inhibitor. Preferably, the proteasome inhibitor is selected from the group consisting of peptidyl aldehydes, boronic acids, boronic esters, lactacystin, and lactacystin analogs. In a preferred embodiment, the proteasome inhibitor is lactacystin or a lactacystin analog, more preferably lactacystin, *clasto*-lactacystin β-lactone, 7-ethyl-*clasto*-lactacystin β-lactone, 7-*n*-propyl-*clasto*-lactacystin β-lactone, or 7-*n*-butyl-*clasto*-lactacystin β-lactone. Most preferably, the proteasome inhibitor is 7-*n*-propyl-*clasto*-lactacystin β-lactone.

In other embodiments of the invention, the agent is a ubiquitin pathway inhibitor.

In yet other embodiments of the invention, the agent is one that interferes with the activation of NF-κB by the ubiquitin-proteasome pathway. Preferably the agent that interferes with the activation of NF-κB is an agent that inhibits phosphorylation of IκB-α.

The invention is further directed to methods for treating a patient afflicted with asthma comprising administering to said patient an effective combination of a glucocorticoid and an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the

ubiquitin proteasome pathway, and mixtures thereof.

In a preferred embodiments, the combination comprises an amount of the agent sufficient to reduce the dose or treatment frequency required for the glucocorticoid. In certain preferred embodiments, the combination comprises an amount of the glucocorticoid that is less than its standard recommended dosage. In another preferred aspect, the combination comprises an amount of the glucocorticoid sufficient to reduce the dose or treatment frequency required for the agent.

In certain preferred embodiments, the glucocorticoid is selected from the group consisting of flunisolide, triamcinolone acetonide, beclomethasone dipropionate, dexamethasone sodium phosphate, fluticasone propionate, budesonide, hydrocortisone, prednisone, prednisolone, mometasone, tipredane, and butixicort.

In other preferred embodiments, the combination used to treat a patient afflicted with asthma comprises a glucocorticoid and a proteasome inhibitor. More preferably, the proteasome inhibitor is lactacystin or a lactacystin analog. Most preferably the combination comprises 7-*n*-propyl-*clasto*-lactacystin β-lactone and budesonide.

The invention is further directed to pharmaceutical compositions comprising a combination of a glucocorticoid and an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, or mixtures thereof. In certain embodiments, the pharmaceutical composition is provided in a unit dosage form. Preferably the unit dosage form comprises the glucocorticoid in an amount that is less than its standard recommended dosage.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of mean clinical score as a function of time in an experimental autoimmune encephalomyelitis model. These data demonstrate that **3b** (7-*n*-propyl-*clasto*-lactacystin β-lactone) treatment causes a reduction in relapse rate and in mean clinical score as compared to vehicle-treated animals.

Figure 2 is a graphical representation of relapse rate as a function of time in an experimental autoimmune encephalomyelitis model. These data demonstrate that **3b** treatment causes a reduction in the rate and severity of relapse.

Figure 3 is a graphical representation of leukocyte count in bronchoalveolar lavage fluid from naive (N) or actively sensitized (AS) Brown Norway rats 72 hours following exposure to aerosolized ovalbumin (10 mg/mL). Treatment with **3b** causes a dose-dependent reduction in leukocyte influx.

Figure 4 is a graphical representation of eosinophil count in bronchoalveolar lavage fluid from naive (N) or actively sensitized (AS) Brown Norway rats 72 hours following exposure to aerosolized ovalbumin (10 mg/mL). Treatment with **3b** causes a dose dependent inhibition of eosinophilia in this model.

Figure 5 is a graphical representation of leukocyte count in bronchoalveolar lavage fluid from naive, untreated (N); actively sensitized, vehicle-treated (V); or actively sensitized, drug-treated (A-H) Brown Norway rats 72 hours following exposure to aerosolized ovalbumin (10 mg/mL). Treatment with budesonide alone (0.1 mg/kg) or **3b** alone (0.03 or 0.1 mg/kg) was ineffective. However, the combination of budesonide (0.1 mg/kg) with **3b** (0.03 or 0.1 mg/kg) causes a reduction in leukocyte influx in this model. High-dose budesonide (0.5 mg/kg) is efficacious with or without added **3b**.

Figure 6 is a graphical representation of eosinophil count in bronchoalveolar lavage fluid from naive, untreated (N); actively sensitized, vehicle-treated (V); or actively

sensitized, drug-treated (A-H) Brown Norway rats 72 hours following exposure to aerosolized ovalbumin (10 mg/mL). Treatment with budesonide alone (0.1 mg/kg) or **3b** alone (0.03 or 0.1 mg/kg) was ineffective. However, the combination of budesonide (0.1 mg/kg) with **3b** (0.03 or 0.1 mg/kg) causes a reduction in eosinophilia in this model. High-dose budesonide (0.5 mg/kg) is efficacious with or without added **3b**.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention is directed to compositions and methods for treatment of inflammatory and autoimmune diseases. All patent applications, patents and literature references cited herein are hereby incorporated by reference in their entirety. In the case of inconsistencies the present disclosure will prevail.

The invention provides methods for treating a patient afflicted with multiple sclerosis or asthma comprising administering to said patient an effective amount of an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof. It has now been unexpectedly discovered that the ubiquitin-proteasome pathway is a target for treating multiple sclerosis, asthma, and rheumatoid arthritis.

In the present description, the following definitions will be used.

"Treating" shall mean any amelioration of any symptom pursuant to administration of any proteasome inhibitor, ubiquitin pathway inhibitor, or agent that interferes with activation of NF-κB via the ubiquitin proteasome pathway.

"Ubiquitin pathway inhibitor" shall mean any substance which specifically inhibits ubiquitination or the transfer of ubiquitin to proteins.

"Proteasome inhibitor" shall mean any substance which specifically inhibits inhibits the proteasome or the activity thereof.

"Agents that interfere with activation of NF-κB by the ubiquitin-proteasome pathway" shall mean any substance that 1) specifically inhibits the proteasome or the activity thereof; 2) specifically inhibits ubiquitination of IκB-α or p105; or 3) specifically inhibits phosphorylation of IκB-α or p105.

"Specifically inhibits" shall mean interferes with the ability of a protein to mediate its biological function at an inhibitor concentration that is lower than

the concentration of the inhibitor required to produce another, unrelated biological effect. Preferably, the concentration of the inhibitor required for such interference is at least 2-fold lower, more preferably at least 5-fold lower, even more preferably at least 10-fold lower, and most preferably at least 20-fold lower than the concentration required to produce an unrelated biological effect. Such inhibitors can act by any of a variety of mechanisms, including without limitation, interfering with the active site or conformation of the protein, interfering with the ability of the protein to interact with another protein, substrate, or co-factor, either by an effect on the protein itself or on the other protein, substrate, or cofactor, and altering the microenvironment in which the biological function of the protein normally occurs.

In a first aspect, the invention provides methods for treating multiple sclerosis. Multiple sclerosis (MS) is an incurable neurological illness that frequently causes chronic disability. It is widely believed that MS is the result of an autoimmune disorder in a genetically susceptible individual, mediated by autoreactive T cells that migrate into the CNS and initiate the inflammatory demyelinating lesion. The observation that MS is an autoimmune disease is derived in part from systemic abnormalities of immune function seen in patients with the disease, and in part through similarities with experimental autoimmune encephalomyelitis (EAE), which in turn serves as a model for the human disease (Kennedy, *et al. J. Neuroimmunol.* (1987) *16*:345; Arnason, *et al. Neurol. Clin.* (1983) *1*:765; van der Veen, *et al. J. Neuroimmunol.* (1989) *48*:213; Gonatas, *et al. Immunol. Today* (1986) 7:121; Wekerle *Acta Neurol.* (1991) *13*:197).

EAE is a T-cell-mediated inflammatory, autoimmune demyelinating disease of the CNS. The disease can be induced in a number of experimental laboratory animals, including primates, by the injection of whole brain homogenate, a purified preparation of myelin basic protein (MBP), or proteolipoprotein (PLP) in adjuvant. EAE is a

T-cell-mediated disease, and passive transfer of MBP- or PLP-reactive T cells is sufficient to induce disease. Relapsing-remitting experimental autoimmune encephalomyelitis (R-EAE) is induced in SJL/J mice by immunization with the immunodominant epitope on proteolipid protein (PLP139-151) or by the adoptive transfer of PLP139-151–specific $CD4^+$ T cells (McRae, *et al. J. Neuroimmunol* (1992) *38*:229). The clinical disease is characterized by an acute paralytic phase followed by recovery and subsequent relapses. This pattern of relapses and spontaneous recovery in the experimental animal model, which occurs over a period of weeks to months, is very similar to the clinical signs of disease observed in multiple sclerosis (MS) patients over many years.

The method according to this aspect of the invention comprises administering to a patient afflicted with MS an effective amount of an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof. In a preferred embodiment, the agent is administered in an amount sufficient to reduce the frequency or severity of relapse of the disease.

When administered during the remission phase at doses of 0.3 or 1.0 mg/kg i.p., the proteasome inhibitor **3b** reduced the rate and severity of relapse in the R-EAE model (Figs. 1-2).

In a second aspect, the invention provides a method for treating asthma. Asthma is an obstructive lung disorder characterized by airway hyperresponsiveness, which is an exaggerated airway narrowing in response to many different stimuli, such as histamine, exercise, cold air, and allergen. Because of the episodic constriction of the bronchial tubes, treatment has been based partly on bronchodilation by β-adrenergic agonist drugs. More recently, however, it has become appreciated that asthma is a chronic condition of the

airways that involves many types of inflammatory cell and the release of many mediators and neurotransmitters that have multiple effects on the various target cells in the airway. The degree and extent of inflammation in the airway wall are broadly related to the clinical severity of the asthma. The inflammatory response of asthma consists of activation of mast cells resident in the airways, increased numbers of lymphocytes (which are mainly $CD4^+$ T lymphocytes) and an infiltration with eosinophils, which appear to degranulate. Increased total eosinophil count in the peripheral blood is almost invariably present unless suppressed by corticosteroids or sympathomimetic drugs. Sputum examination also reveals eosinophils.

Several animal models have been developed to study pulmonary inflammation with characteristic manifestations of airways eosinophilia. One of the often-used animal models is the ovalbumin sensitized guinea pig (Dunn, *et al. Am. Rev. Respir. Dis.* (1990) *142*:680; Sanjar, *et al. Br. J. Pharmacol.* (1990) *99*:679; Gulbenkian, *et al. Am. Rev. Respir. Dis.* (1990) *142*:680). Selective accumulation of both neutrophils and eosinophils have also been described in acutely sensitized Brown Norway rats (Kips, *et al. Am Rev. Respir. Dis.* (1992) *145*:1306; Richards, *et al. Agents Actions, Suppl. 34* (1991) *34*:359; Chapman, *et al. Am. J. Resp. Crit. Care Med.* (1996) *153*:A219). The allergen-induced pulmonary eosinophilia in actively sensitized Brown Norway rats is inhibited by the steroid dexamethasone. Glucocorticoid therapy remains one of the most effective anti-inflammatory treatments available, and these drugs have been shown to reduce pulmonary eosinophilia in asthmatic patients (Holgate, *et al. Int. Arch. Allergy Appl. Immunol.* (1991) *94*:210).

The method according to this aspect of the invention comprises administering to a patient afflicted with asthma an effective amount of an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures

thereof. In a preferred embodiment, the agent is administered in an amount sufficient to reduce the frequency or severity of asthmatic attacks.

When administered intratracheally at 1 hour prior to and 24 hours and 48 hours after allergen challenge, **3b** (0.1 or 0.3 mg/kg) inhibited eosinophilia in actively sensitized Brown Norway rats (Figs. 3-4).

Further contemplated within the scope of the invention is combined administration with another drug or drugs used to treat asthma. Currently accepted therapies for asthma include cromoglycate, nedocromil, theophylline, short- and long-acting β_2-adrenergic receptor agonists, and inhaled or oral glucocorticoids. More recently developed therapeutics include inhibitors of leukotriene biosynthesis, leukotriene receptor antagonists, and thromboxane antagonists. Anti-IL-5 and anti-IgE antibodies are being developed (*Science* (1997) *276*:1643), and antisense approaches are also being investigated (Nyce and Metzger, *Nature* (1997) *383*:721). In one preferred embodiment, the agent of the invention is used in an amount sufficient to reduce the dose or treatment frequency required for the other drug or drugs. In another preferred embodiment, the other drug or drugs are used in an amount sufficient to reduce the dose or treatment frequency required for the agent of the invention. The agent may be administered at the same time as the other drug or drugs or may be administered at a different time.

Steroid therapy is particularly effective for the treatment of asthma, and is an essential line of therapy for severe asthmatics. Unfortunately, however, a number of untoward side-effects result from long-term steroid use, including bone growth suppression, adrenal insufficiency, Cushing's syndrome, cataracts, immunosuppression, and excessive bruising. Many of these effects can be minimized by topical administration of the drug to the lung by inhalation. However, high doses, such as those required in severe cases, result in significant systemic exposure and an increase in the associated

side-effects. Drugs that permit the reduction in steroid dose ("steroid-sparing") thus offer very real clinical benefit.

Unexpectedly, it has been found that intratracheal administration of **3b** (0.03 or 0.1 mg/kg) in combination with the glucocorticoid budesonide (0.1 mg/kg) at 1 hour prior to and 24 hours and 48 hours after allergen challenge inhibits eosinophilia in actively sensitized Brown Norway rats (Figs. 5-6). Strikingly, neither drug was effective when administered alone at these doses, suggesting synergistic action of the two drugs.

In a third aspect, the invention provides methods for treating a patient afflicted with asthma comprising administering to the patient a combination of a glucocorticoid and an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof. The glucocorticoid and the agent may be administered at the same or different times, on the same or different days, and with the same or different frequency. Preferably, the doses of each drug are spaced so as to achieve a combined physiological effect. Preferably, the glucocorticoid is administered between 0 minutes and about one month before or after the agent of the invention, more preferably between 0 minutes and about one week before or after the agent of the invention, most preferably between 0 minutes and 24 hours before or after the agent of the invention.

Glucocorticoids for use in the invention include, but are not limited to, flunisolide, triamcinolone acetonide, beclomethasone dipropionate, dexamethasone sodium phosphate, fluticasone propionate, budesonide, hydrocortisone, prednisone, prednisolone, mometasone, tipredane, and butixicort. Preferably, the glucocorticoid is budesonide. Suitable formulations, dosages, and routes of administration for glucocorticoids are known in the art (*Physician's Desk Reference, 51st Edition*, **1997**, Medical Economics: Montvale, NJ).

In certain preferred embodiments, the agent of the invention is administered in an amount sufficient to reduce the dose or treatment frequency required for the glucocorticoid. Preferably, the amount of glucocorticoid administered does not exceed the standard recommended dosage, and more preferably the amount of glucocorticoid administered is less than the standard recommended dosage for the drug when administered alone.

In certain preferred embodiments, the amount of glucocorticoid administered is sufficient to reduce the dose or treatment frequency required for the agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof. Most preferably, treatment of a patient afflicted with asthma with a combination of a glucococorticoid and an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof produces efficacy with fewer or less severe side effects or toxicity than treatment with either drug alone.

In a fourth aspect, the invention provides pharmaceutical compositions comprising a combination of a glucocorticoid and an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof, are further contemplated within the scope of the invention. The pharmaceutical compositions of the invention can be provided in unit dosage form. In a preferred embodiment, the unit dosage form contains an amount of glucocorticoid that is less than its standard recommended dosage when administered by itself. The following description of non-limiting examples of suitable proteasome inhibitors, ubiquitin pathway inhibitors,

and agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, applies to the pharmaceutical formulations as well as to the methods according to the invention.

Non-limiting examples of proteasome inhibitors for use in the present invention include peptidyl aldehydes (Orlowski et al. U.S. Patent 5,580,854; Stein *et al.* WO 95/24914; Siman *et al.* WO 91/13904; Iqbal *et al. J. Med. Chem. 38*:2276-2277 (1995)), peptidyl boronic acids (Adams *et al.* WO 96/13266; Siman *et al.* WO 91/13904), other peptidyl derivatives with proteasome inhibitory activity (Iqbal et al. U.S. Patent 5,614,649; Iqbal et al. U.S. Patent 5,550,262; Spaltenstein et al. *Tetrahedron Letters* **1996**, *37*, 1343), and lactacystin and lactacystin analogs (Fenteany *et al. Proc. Natl. Acad. Sci. USA* (1994) *91*:3358; Fenteany *et al.* WO 96/32105; Soucy *et al.* U.S. patent application serial no. 08/912,111, filed 8/15/97)). The agents disclosed herein may be administered by any route, including intradermally, intraperitoneally, intranasally, intratracheally, subcutaneously, orally or intravenously. For asthma indications, administration is preferably by the inhalation route.

Peptide aldehyde proteasome inhibitors for use in the present invention preferably are those disclosed in Stein *et al.* WO 95/24914 published September 21, 1995 or Siman *et al.* WO 91/13904 published September 19, 1991, both hereby incorporated by reference in their entirety.

Boronic acid or ester compounds for use in the present invention preferably include those disclosed in Adams *et al.* WO 96/13266, Siman *et al.* WO 91/13904, or Iqbal *et al.* U.S. Pat. 5,614,649, each of which is hereby incorporated by reference in its entirety.

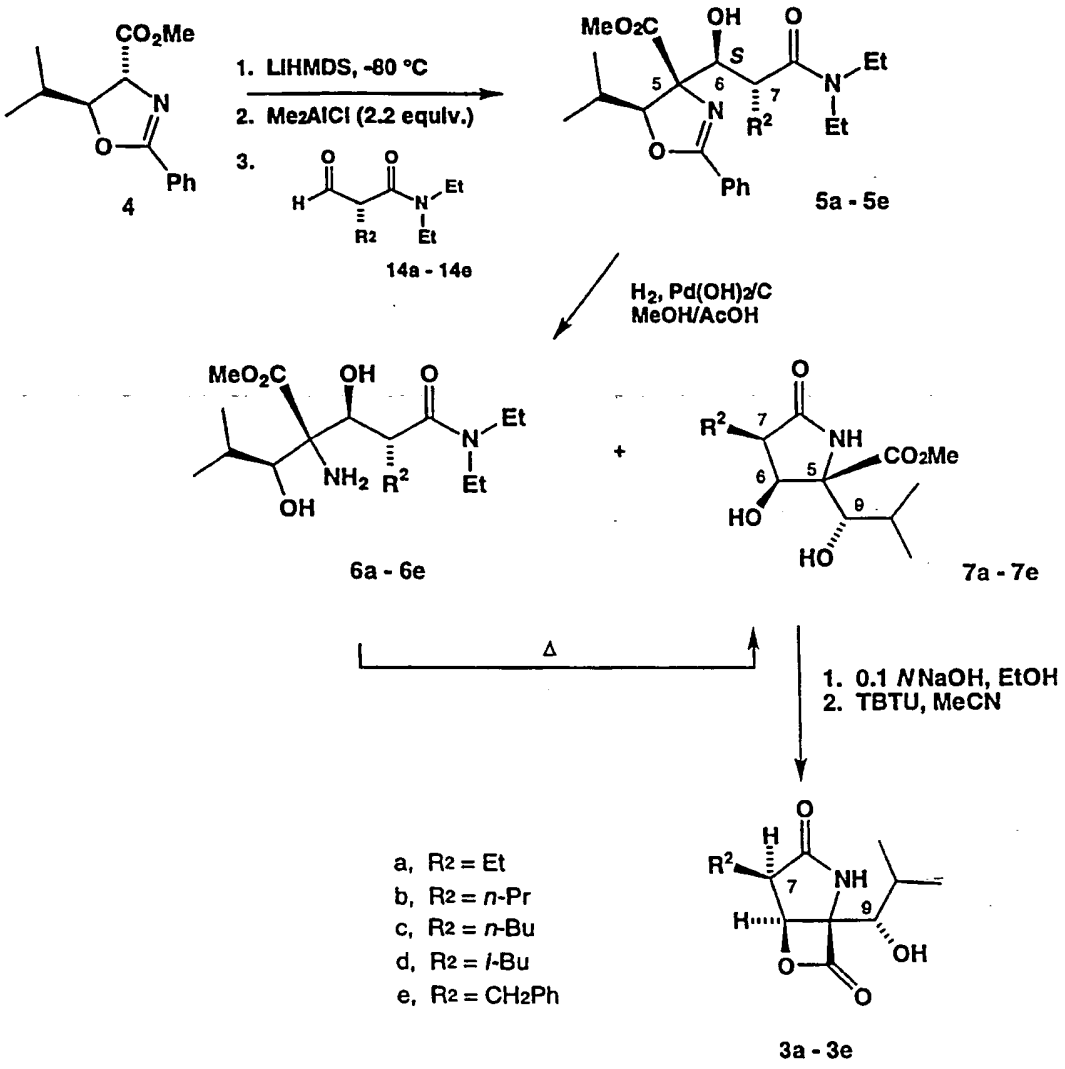
In certain preferred embodiments, the boronic acid compound for use in the present invention is selected from the group consisting of : N-acetyl-L-leucine-ß-(1-

napthyl) L-alanine-L-leucine boronic acid, ß-(1-napthyl)-L-alanine-L-leucine boronic acid, *N*-(4-morpholine)carbonyl-β-(1-naphthyl)-L-alanine-L-leucine boronic acid, *N*-(8-quinoline)sulfonyl-β-(1-naphthyl)-L-alanine-L-leucine boronic acid, *N*-(2-pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid, and *N*-(4-morpholine)carbonyl-[*O*-(2-pyridylmethyl)]-L-tyrosine-L-leucine boronic acid.

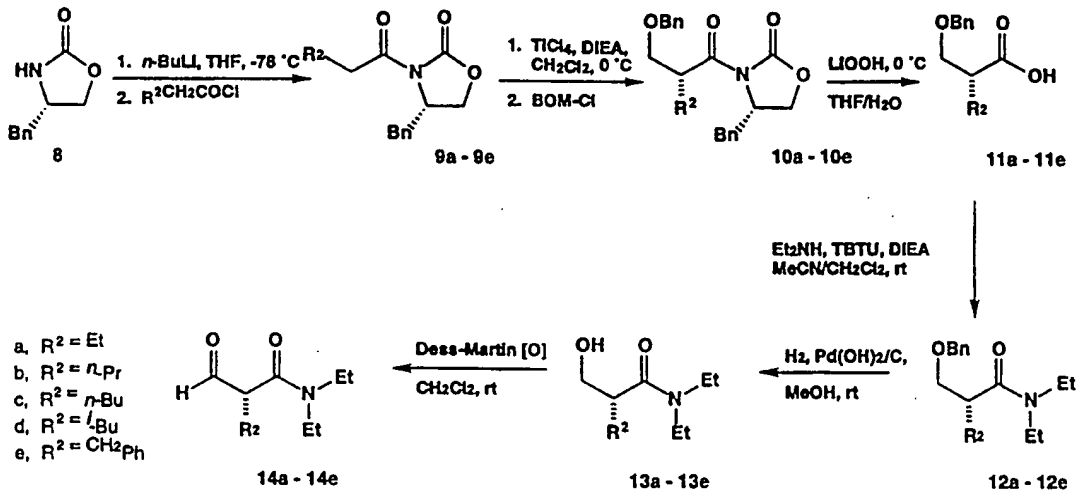
Lactacystin and lactacystin analog compounds for use in the present invention preferably include those disclosed in Fenteany *et al.* WO 96/32105, or Soucy *et al.* U.S. patent application (08/912,111; filed 08/15/97), each of which is hereby incorporated by reference in its entirety. In certain preferred embodiments, the lactacystin analog compound is selected from the group consisting of lactacystin, *clasto*-lactacystin β-lactone, 7-ethyl-*clasto*-lactacystin β-lactone, 7-*n*-propyl-*clasto*-lactacystin β-lactone, and 7-*n*-butyl-*clasto*-lactacystin β-lactone. These compounds can be prepared as illustrated in Schemes 1 and 2. Most preferably, the lactacystin analog compound is 7-*n*-propyl-*clasto*-lactacystin β-lactone (**3b** (Scheme 2)).

In a preferred embodiment, the agent used to treat a patient afflicted with multiple sclerosis or asthma is a proteasome inhibitor. Preferably the proteasome inhibitor is lactacystin or a lactacystin analog, more preferably 7-*n*-propyl-*clasto*-lactacystin β-lactone. Preferably, the combination used to treat a patient afflicted with asthma comprises a glucocorticoid and a proteasome inhibitor. More preferably, the proteasome inhibitor is lactacystin or a lactacystin analog. Most preferably the combination comprises 7-*n*-propyl-*clasto*-lactacystin β-lactone and budesonide.

Scheme 2


CO2Me
N
O
Ph
4
1. LIHMDS, -80 °C
2. Me2AlCl (2.2 equiv.)
3.
O
O
H
N
Et
R2
Et
14a - 14e
MeO2C
OH
O
S
5
6
7
N
Et
R2
O
Ph
5a - 5e
H2, Pd(OH)2/C
MeOH/AcOH
MeO2C
OH
O
N
Et
NH2
R2
Et
OH
6a - 6e
+
O
R2
7
NH
CO2Me
6
5
HO
9
HO
7a - 7e
Δ
1. 0.1 N NaOH, EtOH
2. TBTU, MeCN
a, R2 = Et
b, R2 = n-Pr
c, R2 = n-Bu
d, R2 = i-Bu
e, R2 = CH2Ph
O
H
R2
7
NH
9
H
OH
O
O
3a - 3e

Scheme 1


1. n-BuLi, THF, -78 °C
2. R²CH₂COCl
1. TiCl₄, DIEA, CH₂Cl₂, 0 °C
2. BOM-Cl
LiOOH, 0 °C
THF/H₂O
Et₂NH, TBTU, DIEA
MeCN/CH₂Cl₂, rt
H₂, Pd(OH)₂/C,
MeOH, rt
Dess-Martin [O]
CH₂Cl₂, rt
8
9a - 9e
10a - 10e
11a - 11e
12a - 12e
13a - 13e
14a - 14e
a, R² = Et
b, R² = n-Pr
c, R² = n-Bu
d, R² = i-Bu
e, R² = CH₂Ph

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Non-limiting examples of ubiquitin pathway inhibitors include those disclosed in Berleth et al, *Biochem. 35(5)*:1664-1671, (1996). Inhibitors of IκB-α phosphorylation are also known (Chen, *Cell 84*:853 (1996); Chen U.S. patent application 08/825,559).

The concentration of a disclosed compound in a pharmaceutically acceptable mixture will vary depending on several factors, including the dosage of the compound to be administered, the pharmacokinetic characteristics of the compound(s) employed, and the route of administration. Effective amounts of agents for treating multiple sclerosis, asthma, or rheumatoid arthritis would broadly range between about 10 μg and about 50 mg per Kg of body weight of a recipient mammal. The agent may be administered in a single dose or in repeat doses. Treatments may be administered daily or more frequently depending upon a number of factors, including the age and overall health of a patient, and the formulation and route of administration of the selected compound(s). Other factors to be considered in determining dosage include kind of concurrent treatment, if any; frequency of treatment and the nature of the effect desired; extent of tissue damage; gender; duration of symptoms; counter indications, if any; and other variables to be assessed by the individual physician.

In certain preferred embodiments, the concentration of the proteasome inhibitor is determined by measuring the activity of the proteasome activity *ex vivo* after administering the proteasome inhibitor to the mammal. Such measurement comprises obtaining one or more test biological samples from the mammal at one or more specified times after administering the proteasome inhibitor; measuring proteasome activity in the test biological sample or samples; determining the amount of proteasome activity in the test biological sample or samples; and comparing the amount of proteasome activity in the test biological sample to that in a reference biological sample obtained from a mammal to which no proteasome inhibitor has been administered.

The biological samples that are obtained from the mammal may include, without limitation, blood, urine, organ, and tissue samples. In certain preferred embodiments, the

biological sample is a blood sample, more preferably a blood sample from which the white blood cells are isolated prior to measuring proteasome activity. Methods for fractionating blood cells are known in the art and are further described in the Examples. Hemoglobin strongly interferes with fluorescence measurements and is thus preferably excluded from test samples when fluorometric assays for proteasome activity are used. Once fractionated, the white blood cells are lysed by standard procedures. Variability in lysis efficiency can be corrected for by determining total protein content in the sample using standard procedures and normalizing the proteasome activity measured in the sample relative to the protein content in the sample.

Preferably, a substrate having a detectable label is provided to the reaction mixture and proteolytic cleavage of the substrate is monitored by following disappearance of the substrate or appearance of a cleavage product. Detection of the label may be achieved, for example, by fluorometric, colorimetric, or radiometric assay. More preferably, the substrate is a peptide substrate and the reaction mixture further comprises a 20S proteasome activator. Preferably, the activator is one taught in Coux *et al.* (Ann. Rev. Biochem. 65: 801- 847 (1995)), more preferably PA28 or sodium dodecyl sulfate (SDS). Preferably, the peptide substrate contains a cleavable fluorescent label and release of the label is monitored by fluorometric assay. More preferably, the substrate is *N*-succinylleucylleucylvalyltyrosyl 7- amino-4-methylcoumarin (Suc-Leu-Leu-Val-Tyr-AMC).

The activity measured in the test biological sample is compared to that measured in a reference biological sample obtained from a mammal to which no inhibitor has been administered. In some preferred embodiments, the test biological sample and the reference biological sample each separately comprise a plurality of samples pooled from a group of mammals, preferably mice, undergoing treatment. In other preferred embodiments, the test biological sample and the reference biological sample each comprise a single sample obtained from an individual mammal. Assaying of individual samples is presently preferred except when impractical due to the small size of the mammal. In some preferred embodiments, a statistical sample is obtained by pooling data from individual test biological samples or from individual reference biological samples.

Day-to-day variability in the assay may result from factors such as differences in buffer solutions, operator variability, variability in instrument performance, and temperature variability. Such variability can be minimized by standardizing proteasome

activity in both the biological sample and the reference sample relative to a standard proteasome sample. In certain preferred embodiments, the standard sample comprises purified 20S proteasome, more preferably purified 20S proteasome from rabbit reticulocytes.

In some preferred embodiments, the reference sample is obtained from the treated mammal prior to initiation of treatment. This embodiment is presently preferred for higher mammals in order to minimize the impact of inter-mammal variability. Clinical monitoring of drug action presently preferably entails this embodiment of the invention, with each patient serving as his or her own baseline control.

A decrease in inhibitor activity in the biological sample as compared to the reference sample is indicative of an in vivo effect of the inhibitor at the time the biological sample was obtained. In some preferred embodiments, biological samples are obtained at multiple timepoints following administration of the inhibitor. In these embodiments, measurement of proteasome activity in the biological samples provides an indication of the extent and duration of in vivo effect of the inhibitor. In certain other preferred embodiments, multiple biological samples are obtained from a single mammal at one or more time points. In these embodiments, measurement of proteasome activity in the biological samples provides an indication of the distribution of the inhibitor in the mammal.

In certain preferred embodiments, the dose amount and dose frequency of the proteasome inhibitor are selected so as to avoid excessive proteasome inhibition. In some embodiments, excessive proteasome inhibition results in a toxic effect, the toxic effect including, but not being limited to, vomiting, diarrhea, hypovolemia, hypotension, and lethality. Preferably the dose amount and dose frequency of the proteasome inhibitor are selected so that proteasome inhibition in any future biological sample does not exceed about 95%.

In certain other preferred embodiments, the dose amount and dose frequency of the proteasome inhibitor are selected so that therapeutically useful proteasome inhibition is achieved. Preferably, therapeutically useful proteasome inhibition results in a therapeutically beneficial antitumor, antiinflammatory, antiviral, or antiparasitic effect. Preferably, the dose amount and dose frequency of the proteasome inhibitor are selected so that proteasome inhibition of at least about 15%, preferably about 20%, more preferably about 30%, even more preferably about 40%, and still more preferably about 50%, and most preferably from about 50% to about 80% is achieved in a future biological sample, although in some instances proteasome inhibition as high as 95% may be preferred.

Agents for use in this invention may be prepared for administration by any of the

methods well known in the pharmaceutical art, for example, as described in *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, PA, 1980). Agents may be prepared for use in parenteral administration in the form of solutions or liquid suspensions; for oral administration in the form of tablets or capsules; for intranasal or intratracheal administration in the form of powders, gels, oily solutions, nasal drops, aerosols, or mists. Formulations for parenteral administration may contain as common excipients sterile water or sterile saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Controlled release of an agent may be obtained, in part, by use of biocompatible, biodegradable polymers of lactide, and copolymers of lactide/glycolide or polyoxyethylene/polyoxypropylene. Additional parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration may contain lactose, polyoxyethylene-9-lauryl ether, glycocholate, or deoxycholate.

For the treatment of asthma, the inhalation route of administration is preferred in order to minimize potential side effects or toxicity resulting from systemic exposure to the agent.

According to the present invention, an "effective amount" an agent is an amount sufficient to produce any amelioration of any symptom or sign of the disease (Stites *et al.* *Basic & Clinical Immunology* Lange Medical Publications, Los Altos, California, 1982).

The use of any of the agents disclosed herein in combination with another agent or agents used in the treatment of multiple sclerosis or asthma is further contemplated within the scope of the present invention.

The invention is further exemplified by the following non-limiting examples:

EXAMPLES

Example 1: Relapsing-Remitting Experimental Autoimmune Encephalomyelitis

Materials and Methods

Mice. Female SJL/J mice, 6 weeks old, were purchased from Harlan Laboratories (Indianapolis, IN), housed in the Northwestern animal care facility, and maintained on standard laboratory food and water ad libitum. Paralyzed mice were afforded easier access to food and water.

Peptides. PLP139-151 (HSLGKWLGHPDKF) was purchased from Peptides International (Louisville, KY). Amino acid composition was verified by mass spectrometry and purity (>98%) was assessed by HPLC.

Induction of R-EAE. Mice were immunized by subcutaneous injection of PLP139-151 in complete Freund's adjuvant (CFA) as previously described (McRae, *et al. J. Neuroimmunol.* (1992) *38*:229). Each mouse received 50 μg of PLP139-151 distributed over 2 sites on each hind flank.

Drug Treatment. Starting on day 22, animals (10 per group) were dosed once daily i.p. (5 mL/kg) with vehicle or with **3b** (0.3 or 1.0 mg/kg). Treatment continued through day 40.

Clinical Evaluation. Mice were observed daily for clinical signs of disease. Mice were scored according to their clinical severity as follows: grade 0, no abnormality; grade 1, limp tail; grade 2, limp tail and hind limb weakness (waddling gait); grade 3, partial hind limb paralysis; grade 4, complete hind limb paralysis; and grade 5, moribund.

Results

Data are plotted as the mean daily clinical score for all animals in a particular treatment group (Fig. 1). A relapse was defined as an increase of at least one full grade in clinical score after the animal had previously improved at least a full clinical score and had stabilized. Animals treated with **3b** (both dosage groups) showed reduced clinical scores as compared to vehicle-treated animals. The incidence of relapse was 5/10 for the 0.3 mg/kg group and 2/10 for the 1.0 mg/kg group, as compared to 6/10 for the vehicle-treated group.

Data are also plotted as mean daily relapse incidence for all animals in a particular treatment group (Fig. 2). The mean maximal clinical score per group is also provided as

an indication of disease severity. Animals treated with **3b** (both dosage groups) showed reduced rate of relapse and reduced severity of disease as compared with vehicle-treated animals.

Example 2: Effect of Treatment With 3b on Allergen-Induced Pulmonary Leukocyte Accumulation in Actively Sensitized Brown Norway Rats

Materials and Methods

Rats. Male Brown Norway rats were supplied by Harlan Olac Limited (Bicester, Oxon, UK) and delivered within the weight range of 180-200 g. Following acclimatization for at least five days, animals were actively sensitized over a 3-week period and were within the weight range 250-300 g at the time of allergen exposure. Food and water were provided *ad libitum*.

Sensitization. Ovalbumin (OA; 10 μg) mixed with aluminum hydroxide gel (10 mg) will be injected (0.5 mL, i.p.) into Brown Norway rats and repeated 7 and 14 days later.

Drug Treatment. On day 21, sensitized rats were anaesthetized (halothane 5% in O_2) and **3b**, dexamethasone, or vehicle (lactose) was instilled via a cannula placed directly into the trachea at 1 hour prior to OA exposure. This procedure was repeated at 24 hours and 48 hours after OA exposure.

Challenge. Following recovery, sensitized animals were restrained in plastic tubes and exposed (60 min) to an aerosol of OA (10 mg/mL) using a nose-only exposure system. Animals were sacrificed 72 hours later with pentobarbital (250 mg/kg i.p.).

Analysis. The lungs were lavaged using 3 aliquots (4 mL) of Hank's solution (HBSS × 10, 100 mL; EDTA 100 mM, 100 mL; HEPES 1M, 10 mL made to 11 mL with water); recovered cells were pooled and the total volume of recovered fluid was adjusted to 12 mL by addition of Hank's solution. Total cells were counted (Sysmex Microcell Counter F-500, TOPA Medical Electronics Ltd., Japan). Smears were made by diluting recovered fluid (to approximately 10^6 cells/mL) and spinning an aliquot (100 μL) in a

centrifuge (Cytospin, Shandon, UK). Smears were air dried, fixed using a solution of fast green in methanol (2 mg/L) for 5 seconds and stained with eosin G (5 seconds) and thiazine (5 seconds) (Diff-Quik, Baxter Dade Ltd, Switzerland) in order to differentiate eosinophils, neutrophils, macrophages and lymphocytes. A total of 500 cells per smear were counted by light microscopy under oil immersion (× 1000).

Results

Ovalbumin challenge resulted in a significant increase in eosinophils, neutrophils, and total leukocytes in BAS fluid from actively sensitized (AS) Brown Norway rats as compared to naive (N) rats. Treatment with dexamethasone (0.1 mg/kg i.t.) prevented this increase. At doses of 0.1 and 0.3 mg/kg, **3b** reduced the influx of eosinophils and total leukocytes. A significant decrease in lymphocyte count was also observed at all doses (Figs. 3-4).

Conclusion

Compound **3b** is effective in preventing leukocyte influx following allergen challenge in an animal model of asthma.

Example 3: Effect of Treatment with a Combination of **3b** and Budesonide on Allergen-Induced Pulmonary Leukocyte Accumulation in Actively Sensitized Brown Norway Rats

Materials and Methods

Rats. Male Brown Norway rats were supplied by Harlan Olac Limited (Bicester, Oxon, UK) and delivered within the weight range of 180-200 g. Following acclimatization for at least five days, animals were actively sensitized over a 3-week period and were within the weight range 250-300 g at the time of allergen exposure. Food and water were provided *ad libitum*.

Sensitization. Ovalbumin (OA; 10 μg) mixed with aluminum hydroxide gel (10 mg) will be injected (0.5 mL, i.p.) into Brown Norway rats and repeated 7 and 14 days later.

Drug Treatment. On day 21, sensitized rats were anaesthetized (halothane 5% in O_2) and treated intratracheally (i.t.) 1 hour prior to OA exposure with vehicle (group V; lactose, 1 mg), budesonide (group C, 0.1 mg/kg; group F, 0.5 mg/kg), **3b** (group A, 0.03 mg/kg; group B, 0.1 mg/kg), or mixtures of budesonide and **3b** (group D, 0.1/0.03 mg/kg; group E, 0.1/0.1 mg/kg; group G, 0.5/0.03 mg/kg; group H, 0.5/0.1 mg/kg). Drug was instilled via a cannula placed directly into the trachea. This procedure was repeated at 24 hours and 48 hours after OA exposure.

Challenge. Following recovery, sensitized animals were restrained in plastic tubes and exposed (60 min) to an aerosol of OA (10 mg/mL) using a nose-only exposure system. Animals were sacrificed 72 hours later with pentobarbital (250 mg/kg i.p.).

Analysis. The lungs were lavaged using 3 aliquots (4 mL) of Hank's solution (HBSS × 10, 100 mL; EDTA 100 mM, 100 mL; HEPES 1M, 10 mL made to 11 mL with water); recovered cells were pooled and the total volume of recovered fluid was adjusted to 12 mL by addition of Hank's solution. Total cells were counted (Sysmex Microcell Counter F-500, TOPA Medical Electronics Ltd., Japan). Smears were made by diluting recovered fluid (to approximately 10^6 cells/mL) and spinning an aliquot (100 μL) in a centrifuge (Cytospin, Shandon, UK). Smears were air dried, fixed using a solution of fast green in methanol (2 mg/L) for 5 seconds and stained with eosin G (5 seconds) and thiazine (5 seconds) (Diff-Quik, Baxter Dade Ltd, Switzerland) in order to differentiate eosinophils, neutrophils, macrophages and lymphocytes. A total of 500 cells per smear were counted by light microscopy under oil immersion (× 1000).

Results

Ovalbumin challenge resulted in a significant increase in eosinophils, neutrophils, and total leukocytes in BAS fluid from actively sensitized, vehicle treated (V) Brown

Norway rats as compared to naive, untreated (N) rats. At doses of 0.03 mg/kg (A) and 0.1 mg/kg (B), **3b** failed to prevent this increase. At a dose of 0.1 mg/kg (C), budesonide also had not effect when administered alone. However, the combination of 0.1 mg/kg budesonide with **3b** at 0.03 mg/kg (D) or 0.1 mg/kg (E) produced a significant reduction in eosinophil count. Statistically significant reduction in neutrophil count was achieved only in the 0.1/0.03 mg/kg (D) group. At higher dose (0.5 mg/kg, group F) budesonide treatment alone was effective in preventing the increase in eosinophils, neutrophils, and total leukocytes, and combination of budesonide (0.5 mg/kg) with 0.03 mg/kg (G) or 0.1 mg/kg (H) of **3b** was also efficacious (Figs. 5-6).

Conclusion

The combination of compound **3b** with the glucocorticoid budesonide is effective in preventing leukocyte influx following allergen challenge in an animal model of asthma at doses where neither drug alone has any effect.

Example 4: Preparation of formyl amides 14 (Scheme 1)

Acyl oxazolidinone 9b (R= *n*-Pr)

A cooled (-78 °C) solution of (*S*)-(-)-4-benzyl-2-oxazolidinone (4.0 g, 22.6 mmol) in 75 mL anhydrous THF was treated with a 2.5 M solution of *n*-BuLi in hexane (9.1 mL, 22.6 mmol) over 15 min. After 5 min, neat valeryl chloride (2.95 mL, 24.9 mmol) was added dropwise and the mixture was stirred for another 45 min. at -78 °C. The mixture was then allowed to reach room temperature, stirred for another 90 min, and then treated with 50 mL saturated NH_4Cl solution. Dichloromethane (50 mL) was then added and the organic layer was washed with brine (2 x 30 mL), dried over $MgSO_4$ and concentrated *in vacuo*. This afforded 5.94 g (100 %) of the desired acyl oxazolidinone **9b** as a clear colorless oil. ^1H NMR (300 MHz, $CDCl_3$) δ 7.36-7.20 (m, 5H), 4.71-4.64 (m, 1H), 4.23-4.14 (m, 1H), 3.40 (dd, *J*= 13.3, 3.2 Hz, 1H), 3.04-2.84 (m, 2H), 2.77 (dd, *J*=13.3, 9.6 Hz, 1H);

1.74-1.63 (m, 2H), 1.46-1.38 (m, 2H), 0.96 (t, *J*=7.3 Hz, 3H).

Acyl oxazolidinone 9a (R= Et)

By a procedure analogous to that described for preparing acyl oxazolidinone **9b**, the lithium anion of (*S*)-(-)-4-benzyl-2-oxazolidinone was treated with butyryl chloride to provide acyl oxazolidinone **9a** in 94% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37-7.20 (m, 5H), 4.68 (ddd, *J*= 13.1, 7.0, 3.4 Hz, 1H), 4.23-4.13 (m, 2H), 3.30 (dd, *J*= 13.3, 9.6 Hz, 1H), 3.02-2.82 (m, 2H), 2.77 (dd, *J*=13.3, 9.6 Hz, 1H), 1.73 (q, *J*= 7.3 Hz, 2H), 1.01 (t, *J*= 7.3 Hz, 3H).

Acyl oxazolidinone 9c (R= *n*-Bu)

By a procedure analogous to that described for preparing acyl oxazolidinone **9b**, the lithium anion of (*S*)-(-)-4-benzyl-2-oxazolidinone was treated with hexanoyl chloride to provide acyl oxazolidinone **9a** in 96% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36-7.20 (m, 5H), 4.68 (m, 1H), 4.23-4.14 (m, 2H), 3.30 (dd, *J*= 13.3, 3.3 Hz, 1H), 3.02-2.83 (m, 2H), 2.76 (dd, *J*=13.3, 9.6 Hz, 1H), 1.70 (m, 2H), 1.43-1.34 (m, 4H), 0.92 (t, *J*= 3.3 Hz, 3H).

4-Methylvaleryl chloride

4- Methylvaleryl chloride was prepared from commercially available 4-methylvaleric acid in the following way: a cold (0 °C) solution of 4-methylvaleric acid (1.85 mL, 15.0 mmol) in 50 mL anhydrous CH_2Cl_2 containing 10 mL of DMF was treated with 1.95 μL oxalyl chloride (22.5 mmol). The mixture was then stirred for 3 h at room temperature, concentrated *in vacuo* and filtered to affords 1.65 g (100%) of the desired acid chloride as a colorless liquid.

Acyl oxazolidinone 9d (R= *i*-Bu)

By a procedure analogous to that described for preparing acyl oxazolidinone **9b**, the lithium anion of (*S*)-(-)-4-benzyl-2-oxazolidinone was treated with 4-methylvaleryl chloride to provide acyl oxazolidinone **9d** in 85% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ

7.37-7.20 (m, 5H), 4.70-4.63 (m, 1H), 4.23-4.15 (m, 2H), 3.30 (dd, J= 13.2, 3.2 Hz, 1H), 2.98-2.90 (m, 2H), 2.76 (dd, J=13.3, 9.6 Hz, 1H), 1.68-1.54 (m, 3H), 0.94 (d, J= 6.2 Hz, 3H).

Acyl oxazolidinone 9e (R= CH_2Ph)

By a procedure analogous to that described for preparing acyl oxazolidinone **9b**, the lithium anion of (*S*)-(-)-4-benzyl-2-oxazolidinone was treated with hydrocinnamoyl chloride to provide acyl oxazolidinone **9e** in 82% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35-7.16 (m, 10H), 4.70-4.63 (m, 1H), 4.21-4.14 (m, 2H), 3.38-3.19 (m, 3H), 3.08-2.98 (m, 2H), 2.75 (dd, J=13.4, 9.5 Hz, 1H).

Acyl oxazolidinone 10b (R= *n*-Pr)

A cold (0 °C) solution of acyl oxazolidinone **9b** (5.74 g, 22.0 mmol) in 110 mL anhydrous CH_2Cl_2 was treated with 2.52 mL $TiCl_4$ (23.1 mmol) resulting in the formation of an abundant precipitate. After 5 min, diisopropylethylamine (4.22 mL, 24.2 mmol) was added slowly and the resulting dark brown solution was stirred at room temperature for 35 min. Benzyl chloromethyl ether (6.0 mL, 44.0 mmol) was the rapidly added and the mixture was stirred for 5 h at room temperature. 50 mL CH_2Cl_2 and 75 mL of 10% aqueous NH_4Cl were then resulting in the formation of yellow gummy material. After stirring the suspension vigorously for 10 min, the supernatant was transferred in a separatory funnel and the gummy residue was taken up in 100 mL 1:1 10% aqueous NH_4Cl/CH_2Cl_2. The combined organic layers were then washed successively with 1N aqueous HCl, saturated $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude solid material was recrystallized from EtOAc/hexane affording 6.80 g of desired acyl oxazolidinone **10b** as a white solid in 81% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34-7.18 (m, 10H), 4.77-4.69 (m, 1H), 4.55 (s, 2H), 4.32-4.23 (m, 1H), 4.21-4.10 (m, 2H), 3.80 (t, J= 9.0 Hz, 1H), 3.65 (dd, J= 9.0, 5.0 Hz, 1H), 3.23 (dd, J= 13.5, 3.3 Hz, 1H), 2.69 (dd, J= 13.5, 9.3 Hz, 1H), 1.74-1.64 (m, 1H), 1.54-1.44 (m, 1H), 1.40-1.28 (m, 2H), 0.91 (t, J= 7.3 Hz, 3H).

LRMS (FAB) *m/e* 382 ($M+H^+$)

Acyl oxazolidinone 10a (R= Et)

By a procedure analogous to that described for preparing acyl oxazolidinone **10b**, acyl oxazolidinone **10a** was obtained in 80% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 7.36-7.18 (m, 10H), 4.55 (s, 2H), 4.21-4.11 (m, 3H), 3.81 (t, *J*= 9.0 Hz, 1H), 3.66 (dd, *J*= 9.0, 5.0 Hz, 1H), 3.23 (dd, *J*= 13.5, 3.2 Hz, 1H), 2.70 (dd, *J*= 13.5, 9.3 Hz, 1H), 1.78-1.57 (m, 2H), 0.94 (t, *J*= 7.5 Hz, 3H).

Acyl oxazolidinone 10c (R= *n*-Bu)

By a procedure analogous to that described for preparing acyl oxazolidinone **10b**, acyl oxazolidinone **10c** was obtained in 91% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 7.38-7.17 (m, 10H), 4.72 (m, 1H), 4.54 (s, 2H), 4.27-4.10 (m, 2H), 3.79 (t, *J*= 8.7 Hz, 1H), 3.65 (dd, *J*= 9.1, 5.0 Hz, 1H), 3.23 (dd, *J*= 13.5, 3.3 Hz, 1H), 2.68 (dd, *J*= 13.5, 9.3 Hz, 1H), 1.75-1.68 (m, 1H), 1.31-1.26 (m, 4H), 0.87 (t, *J*= 6.8 Hz, 3H).

Acyl oxazolidinone 10d (R= *i*-Bu)

By a procedure analogous to that described for preparing acyl oxazolidinone **10b**, acyl oxazolidinone **10d** was obtained in 98% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 7.38-7.17 (m, 10H), 4.75-4.67 (m, 1H), 4.57 (d, *J*= 12.0 Hz, 1H), 4.51 (d, *J*= 12.0 Hz, 1H), 4.41-4.36 (m, 1H), 4.20-4.09 (m, 2H), 3.74 (t, *J*= 9.0 Hz, 1H), 3.65 (dd, *J*= 9.0, 5.1 Hz, 1H), 3.23 (dd, *J*= 13.5, 3.2 Hz, 1H), 2.63 (dd, *J*= 13.5, 9.5 Hz, 1H), 1.74-1.52 (m, 2H), 1.35 (dd, *J*= 13.1, 6.1 Hz, 1H), 0.92 (d, *J*= 2.9 Hz, 3H), 0.90 (d, *J*= 2.9 Hz, 3H).

Acyl oxazolidinone 10e (R= CH_2Ph)

By a procedure analogous to that described for preparing acyl oxazolidinone **10b**, acyl oxazolidinone **10e** was obtained in 84% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 7.38-7.15 (m, 15H), 4.62-4.50 (m, 4H), 4.03 (dd, *J*= 9.0, 2.7 Hz, 1H), 3.93-3.82 (m, 2H), 3.66 (dd, *J*= 9.2, 4.8 Hz, 1H), 3.19 (dd, *J*= 13.5, 3.2 Hz, 1H), 2.98 (dd, *J*= 13.4, 8.2 Hz, 1H), 2.88

(dd, *J*= 13.4, 7.3 Hz, 1H), 2.68 (dd, *J*= 13.5, 9.3 Hz, 1H).

Carboxylic acid 11b (R= *n*-Pr)

A cold (0 °C) solution of 6.60 g (17.3 mmol) of acyl oxazolidinone **10b** in 320 mL THF/H_2O was treated successively with 6.95 mL 35% aqueous H_2O_2 and a solution of lithium hydroxide monohydrate (1.46 g, 34.6 mmol) in 20 mL H_2O. The mixture was stirred for 16 h at 0 °C and then treated carefully first with a solution Na_2SO_3 (10.5 g) in 55 mL H_2O and then with a solution of $NaHCO_3$ (4.35 g) in 100 mL H_2O. The mixture was stirred for 30 min at room temperature and concentrated *in vacuo* to remove the THF. The resulting aqueous mixture was then washed with CH_2Cl_2 (4 x 75 mL), cooled to 0 °C, acidified with 6N aqueous HCl and extracted with CH_2Cl_2 (1 x 200 mL and 3 x 100 mL). The combined organic layers were then dried over $MgSO_4$ and concentrated in vacuo affording 3.47 g (90%) of desired acid **11b** as a clear colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38-7.26 (m, 5H), 4.55 (s, 2H), 3.67 (m, 1H), 3.57 (dd, *J*= 9.2, 5.2 Hz, 1H), 2.75 (m, 1H), 1.72-1.31 (m, 4H), 0.93 (t, *J*= 7.2 Hz, 3H).

LRMS (FAB) *m/e* 223 ($M+H^+$)

Carboxylic acid 11a (R= Et)

By a procedure analogous to that described for preparing acyl oxazolidinone **11b**, acyl oxazolidinone **11a** was obtained in 48% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36-7.27 (m, 5H), 4.55 (s, 2H), 3.68 (dd, *J*= 9.2, 7.9 Hz, 1H), 3.59 (dd, *J*= 9.2, 5.4 Hz, 1H), 2.68-2.65 (m, 1H), 1.71-1.62 (m, 2H), 0.97 (t, *J*= 7.5 Hz, 3H).

Carboxylic acid 11c (R= *n*-Bu)

By a procedure analogous to that described for preparing acyl oxazolidinone **11b**, acyl oxazolidinone **11c** was obtained in 96% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37-7.28 (m, 5H), 4.55 (s, 2H), 3.67 (dd, *J*= 9.1, 8.1 Hz, 1H), 3.57 (dd, *J*= 9.2, 5.3 Hz, 1H), 2.72 (m, 1H), 1.67-1.51 (m, 2H), 1.36-1.27 (m, 4H), 0.89 (t, *J*= 6.9 Hz, 3H).

Carboxylic acid 11d (R= *i*-Bu)

By a procedure analogous to that described for preparing acyl oxazolidinone **11b**, acyl oxazolidinone **11d** was obtained in 80% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37-7.28 (m, 5H), 4.55 (s, 2H), 3.64 (t, *J*= 9.1 Hz, 1H), 3.54 (dd, *J*= 9.1, 5.1 Hz, 1H), 2.81 (m, 1H), 1.68-1.54 (m, 2H), 1.36-1.27 (m, 1H), 0.92 (d, *J*= 4.9 Hz, 3H), 0.90 (d, *J*= 4.9 Hz, 3H).

Carboxylic acid 11e (R= CH_2Ph)

By a procedure analogous to that described for preparing acyl oxazolidinone **11b**, acyl oxazolidinone **11e** was obtained in 92% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38-7.16 (m, 10H), 4.53 (d, *J*= 12.1 Hz, 1H), 4.50 (d, *J*= 12.1 Hz, 1H), 3.68-3.57 (m, 2H), 3.09-2.85 (m, 3H).

Diethylamide 12b (R= *n*-Pr)

A cooled solution (0 °C) of carboxylic acid **11b** (3.40 g, 15.3 mmol) in 1:1 MeCN/CH_2Cl_2 (150 mL), containing diethylamine (2.36 mL, 23.0 mmol) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 5.89 g, 18.4 mmol), was treated with diisopropylethylamine (6.7 mL, 38.2 mmol) over 1.5 h (syringe pump). The mixture was then concentrated in vacuo and partitioned between ether (200 mL) and H2O (100 mL). The aqueous layer was extracted with more ether (2 x 100 mL) and the combined organic layers were washed with aqueous 1N HCl (3 x 50 mL), saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Chromatographic purification (230-400 mesh SiO_2, elution with 1:3 AcOEt/hexane) afforded 4.24 g (97%) of diethyl amide **12b** as a clear colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35-7.23 (m, 5H), 4.52 (d, *J*= 12.0 Hz, 1H), 4.44 (d, *J*= 12.0 Hz, 1H), 3.67 (t, *J*= 8.6 Hz, 1H), 3.51 (dd, *J*= 8.7, 5.5 Hz, 1H), 3.46-3.27 (m, 4H), 2.96 (m, 1H), 1.67-1.57 (m, 1H), 1.48-1.22 (m, 4H), 1.20-1.10 (m, 6H), 0.90 (t, *J*= 7.2 Hz, 3H).
LRMS (FAB) *m/e* 278 ($M+H^+$)

Diethylamide 12a (R= Et)

By a procedure analogous to that described for preparing diethylamide **12b**, diethylamide **12a** was obtained in 73% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33-7.26 (m, 5H), 4.52 (d, *J*= 12.0 Hz, 1H), 4.44 (d, *J*= 12.0 Hz, 1H), 3.68 (t, *J*= 8.6 Hz, 1H), 3.53-3.33 (m, 5H), 2.90 (m, 1H), 1.75-1.50 (m, 2H), 1.18 (t, *J*= 7.1 Hz, 3H), 1.13 (t, *J*= 7.1 Hz, 3H), 0.89 (t, *J*= 7.4 Hz, 3H).

Diethylamide 12c (R= *n*-Bu)

By a procedure analogous to that described for preparing diethylamide **12b**, diethylamide **12c** was obtained in 94% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35-7.25 (m, 5H), 4.51 (d, *J*= 12.0 Hz, 1H), 4.44 (d, *J*= 12.0 Hz, 1H), 3.67 (t, *J*= 8.6 Hz, 1H), 3.51 (dd, *J*= 8.8, 5.5 Hz, 1H), 3.46-3.29 (m, 1H), 2.94 (m, 1H), 1.66-1.62 (m, 2H), 1.33-1.10 (m, 9H), 0.85 (t, *J*= 7.0 Hz, 3H).

Diethylamide 12d (R= *i*-Bu)

By a procedure analogous to that described for preparing diethylamide **22b**, diethylamide **12d** was obtained in 95% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35-7.23 (m, 5H), 4.51 (d, *J*= 12.0 Hz, 1H), 4.44 (d, *J*= 12.0 Hz, 1H), 3.65 (t, *J*= 8.7 Hz, 1H), 3.54-3.28 (m, 5H), 3.03 (m, 1H), 1.63-1.49 (m, 2H), 1.33-1.24 (m, 1H), 1.18 (t, *J*= 7.1 Hz, 3H), 1.12 (t, *J*= 7.1 Hz, 3H), 0.90 (t, *J*= 6.4 Hz, 3H).

Diethylamide 12e (R= CH_2Ph)

By a procedure analogous to that described for preparing diethylamide **12b**, diethylamide **12e** was obtained in 89% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35-7.16 (m, 10H), 4.53 (d, *J*= 12.1 Hz, 1H), 4.47 (d, *J*= 12. 1 Hz, 1H), 3.77 (t, *J*= 8.5 Hz, 1H), 3.59 (dd, *J*= 8.8, 5.7 Hz, 1H), 3.40 (m, 1H), 3.22-2.89 (m, 5H), 2.79 (dd, *J*= 13.0, 5.1 Hz, 3H), 1.01 (t, *J*= 7.1 Hz, 3H), 0.85 (t, *J*= 7.2 Hz, 3H).

Alcohol 13b (R= *n*-Pr)

To a solution of diethylamide **12b** (4.08 g, 14.7 mmol) in 140 mL MeOH was added 20% $Pd(OH)_2/C$ (400 mg) and the suspension was hydrogenated at atmospheric pressure and room temperature for 15 h. Filtration of the catalyst and concentrating the filtrate in vacuo afforded 2.84 g (100%) of the desired primary alcohol **13b**. ^1H NMR (300 MHz, $CDCl_3$) δ 3.74 (br. d, *J*=4.2 Hz, 1H), 3.61-3.15 (m, 5H), 2.71 (m, 1H), 1.69-1.24 (m, 4H), 1.20 (t, *J*= 7.1 Hz, 3H), 1.12 (t, *J*= 7.1 Hz, 3H), 0.92 (t, *J*= 7.2 Hz, 3H).
LRMS (FAB) *m/e* 188 ($M+H^+$)

Alcohol 13a (R= Et)

By a procedure analogous to that described for preparing alcohol **13b**, alcohol **13a** was obtained in 100% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 3.76 (m, 2H), 3.58-3.19 (m, 4H), 2.64 (m, 1H), 1.71-1.65 (m, 2H), 1.21 (t, *J*= 7.1 Hz, 3H), 1.13 (t, *J*= 7.1 Hz, 3H), 0.96 (t, *J*= 7.4 Hz, 3H).

Alcohol 13c (R= *n*-Bu)

By a procedure analogous to that described for preparing alcohol **13b**, alcohol **13c** was obtained in 100% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 3.76 (d, J= 4.5 Hz, 2H), 3.58-3.19 (m, 4H), 2.72-2.65 (m, 2H), 1.68-1.55 (m, 2H), 1.40-1.24 (m, 4H), 1.20 (t, *J*= 7.1 Hz, 3H), 1.12 (t, *J*= 7.1 Hz, 3H), 0.90 (t, *J*= 6.9 Hz, 3H).

Alcohol 13d (R= *i*-Bu)

By a procedure analogous to that described for preparing alcohol **13b**, alcohol **13d** was obtained in 100% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 3.78-3.68 (m, 2H), 3.57-3.15 (m, 4H), 2.81-2.73 (m, 1H), 1.70-1.60 (m, 2H), 1.40-1.28 (m, 1H), 1.21 (t, *J*= 7.1 Hz, 3H), 1.12 (t, *J*= 7.1 Hz, 3H), 0.92 (m, 6H).

Alcohol 13e (R=CH_2Ph)

By a procedure analogous to that described for preparing alcohol **13b**, alcohol **13e** was obtained in 100% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 7.29-7.16 (m, 5H), 3.81-3.71 (m,

2H), 3.61-3.50 (m, 1H), 3.15-2.87 (m, 6H), 1.05 (t, *J*= 7.1 Hz, 3H), 0.98 (t, *J*= 7.1 Hz, 3H).

Aldehyde 14b (R= *n*-Pr)

To a solution of alcohol **13b** (2.34 g, 12.7 mmol) in wet CH_2Cl_2 (125 mL, prepared by stirring CH_2Cl_2 with water and separating the organic layer) was added Dess-Martin periodinane (8.06 g, 19.0 mmol). The mixture was stirred at room temperature for 40 min and was then poured into a mixture of 5% aqueous $Na_2S_2O_3$ (250 mL) containing 5.2 g $NaHCO_3$, and ether (200 mL). The biphasic mixture was stirred vigorously for 5 min and the aqueous layer was extracted with 15% CH_2Cl_2/Et_2O (2 x 100 mL). The combined organic layers were then washed with H_2O (3 x 75 mL) and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford 2.06 g (88%) of desired aldehyde **14b**, a clear colorless oil. ^1H NMR (300 MHz, $CDCl_3$) δ 9.60 (d, *J*= 3.5 Hz, 1H), 3.49-3.30 (m, 5H), 1.96-1.85 (m, 2H), 1.39-1.31 (m, 2H), 1.19 (t, *J*=7.1 Hz, 3H), 1.13 (t, *J*= 7.1 Hz, 3H), 0.95 (t, *J*= 7.3 Hz, 3H).

Aldehyde 14a (R= Et)

By a procedure analogous to that described for preparing alcohol **14b**, aldehyde **14a** was obtained in 80% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 9.61 (d, *J*= 3.6 Hz, 1H), 3.48-3.29 (m, 5H), 2.02-1.90 (m, 2H), 1.19 (t, *J*=7.1 Hz, 3H), 1.14 (t, *J*= 7.1 Hz, 3H), 0.96 (t, *J*= 7.4 Hz, 3H).

Aldehyde 14c (R= *n*-Bu)

By a procedure analogous to that described for preparing alcohol **14b**, aldehyde **14c** was obtained in 98% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 9.59 (d, *J*= 3.6 Hz, 1H), 3.48-3.29 (m, 5H), 1.97-1.87 (m, 2H), 1.39-1.22 (m, 4H), 1.18 (t, *J*=7.2 Hz, 3H), 1.13 (t, *J*= 7.2 Hz, 3H), 0.90 (t, *J*= 7.0 Hz, 3H).

Aldehyde 14d (R= *i*-Bu)

By a procedure analogous to that described for preparing alcohol **14b**, aldehyde **14d** was obtained in 96% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.57 (d, *J*= 3.7 Hz, 1H), 3.51-3.27 (m, 5H), 1.83 (t, *J*=7.1 Hz, 3H), 1.66-1.55 (m, 1H), 1.20 (t, *J*= 7.1 Hz, 3H), 1.13 (t, *J*= 7.1 Hz, 3H), 0.93 (d, *J*= 6.6 Hz, 6H).

Aldehyde 14e (R= CH_2Ph)

By a procedure analogous to that described for preparing alcohol **14b**, aldehyde **14e** was obtained in 97% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.69 (d, *J*= 2.9 Hz, 1H), 7.29-7.16 (m, 5H), 3.65 (m, 1H), 3.53-3.42 (m, 1H), 3.30 (dd, *J*= 13.5, 9.3 Hz, 1H), 3.23-3.13 (m, 2H), 3.06-2.91 (m, 2H), 1.04 (t, *J*=7.1 Hz, 3H), 0.93 (t, *J*= 7.1 Hz, 3H).

Example 5: Preparation of β-lactones 3 (Scheme 2)

Aldol 5b (R= *n*-Pr)

To a cold (-78 °C) solution of *trans*-oxazoline **4** in ether (35 mL) was added lithium bis(trimethylsilyl)amide (2.17 of a 1 M solution in hexane, 2.17 mmol). After 30 min, the orange solution was treated dropwise with a 1M solution of dimethylaluminum chloride in hexane (4.55 mL, 4.55 mmol) and the mixture was stirred for another 60 min before being cooled down to -85 °C (liquid N_2 was added to the dry ice/acetone bath). A solution of aldehyde **14b** (420 mg, 2.27 mmol) in ether (4 mL) was the added over 10 min along the side of the flask. The mixture was then allowed to warm up to - 40 °C over 2.5 h and then quenched by adding 35 mL of saturated aqueous NH_4Cl and 25 mL AcOEt. Enough 2 N HCl was then added until 2 clear phases are obtained (*ca.* 15 mL added). The aqueous layer was extracted with AcOEt (2 x 20 mL) and the combined organic layers were washed successively with 0.5 N aqueous HCl (20 mL), H_2O (20 mL), 0.5 M aqueous $NaHSO_3$ (2 x 15 mL), saturated aqueous $NaHCO_3$ and finally with brine, then dried over Na_2SO_4 and concentrated in vacuo affording 879 mg (> 100%) of crude aldol product **5b** which was pure enough to be used directly in the subsequent step. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02-7.97 and 7.53-7.39 (m, 5H), 6.58 (d, *J*= 9.9 Hz, 1H), 4.82 (d, *J*= 2.4 Hz,

1H), 3.73 (s, 3H), 3.69-3.61 (m, 2H), 3.49-3.39 (m, 2H), 3.24-3.16 (m, 1H), 3.05 (m, 1H), 2.89 (m, 1H), 2.28-2.23 (m, 1H), 1.98-1.91 (m, 1H), 1.37-1.20 (m, 6H), 1.19-1.06 (m, 6H), 0.87 (t, *J*= 7.1 Hz, 3H), 0.70 (d, *J*= 6.7 Hz, 3H).

Aldol product **5b** was also obtained in 100% yield by a procedure analogous to that described above but using cis-oxazoline **21** (see below) instead of *trans*-oxazoline **4**.

Aldol 5a (R= Et)

By a procedure analogous to that described for preparing aldol **5b**, the lithium anion of *trans*-oxazoline **4** was treated successively with dimethylaluminum chloride and aldehyde **14a** to provide aldol **5a** in 95% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.00-7.97 and 7.51-7.39 (m, 5H), 6.50 (d, *J*= 9.9 Hz, 1H), 4.80 (d, *J*= 2.4 Hz, 1H), 3.81-3.64 (m, 2H), 3.74 (s, 3H), 3.45 (m, 2H), 3.19 (m, 2H), 2.93-2.84 (m, 2H), 2.24 (m, 1H), 1.89 (m, 1H), 1.73-1.64 (m, 4H), 1.29 (t, *J*= 7.2 Hz, 3H), 1.12 (d, *J*= 6.9 Hz, 3H), 1.07 (d, *J*= 7.2 Hz, 3H), 0.70 (d, *J*= 6.7 Hz, 3H).

Aldol 5c (R= *n*-Bu)

By a procedure analogous to that described for preparing aldol **5b**, the lithium anion of *trans*-oxazoline **4** was treated successively with dimethylaluminum chloride and aldehyde **14c** to provide aldol **5c** in 100% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02-7.98 and 7.53-7.33 (m, 5H), 6.57 (d, *J*= 10.0 Hz, 1H), 4.81 (d, *J*= 2.3 Hz, 1H), 3.73 (s, 3H), 3.68-3.60 (m, 2H), 3.49-3.17 (m, 2H), 3.00 (m, 1H), 2.90 (m, 1H), 1.98-1.87 (m, 2H), 1.38-0.83 (m, 16H), 0.70 (d, *J*= 6.7 Hz, 3H).

Aldol 5d (R= *i*-Bu)

By a procedure analogous to that described for preparing aldol **5b**, the lithium anion of *trans*-oxazoline **4** was treated successively with dimethylaluminum chloride and aldehyde **14d** to provide aldol **5d** in 100% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01-7.80 and 7.55-7.20 (m, 5H), 4.87 (d, *J*= 2.3 Hz, 1H), 3.73 (s, 3H), 3.69-3.58 (m, 2H), 3.51-3.32

(m, 2H), 2.98-2.87 (m, 1H), 2.33-2.24 (m, 1H), 2.12-2.02 (m, 1H), 1.83 (t, J= 7.1 Hz, 1H), 1.35 (t, J= 7.1 Hz, 3H), 1.25-1.05 (m, 5H), 0.93 (d, J= 6.6 Hz, 3H), 0.89 (d, J= 6.5 Hz, 3H), 0.80 (d, J= 6.5 Hz, 3H), 0.69 (d, J= 6.7 Hz, 3H).

Aldol 5e (R= CH_2Ph)

By a procedure analogous to that described for preparing aldol **5b**, the lithium anion of *trans*-oxazoline **4** was treated successively with dimethylaluminum chloride and aldehyde **14e** to provide aldol **5e** in 100% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01-7.93 and 7.54-7.10 (m, 10H), 4.71 (d, J= 2.5 Hz, 1H), 3.73 (s, 3H), 3.68-3.58 (m, 2H), 3.48-2.79 (m, 6H), 2.17 (m, 1H), 1.12-0.91 (m, 9H), 0.68 (d, J= 6.7 Hz, 3H).

γ-Lactam 7b (R= *n*-Pr)

A solution of aldol **5b** (4.72 g, 10.9 mmol) in 100 mL 1:9 AcOH/MeOH, to which was added 4.8 g 20% $Pd(OH)_2/C$, was vigorously shaken under 55 p.s.i. H_2 for 60 h. The mixture was brought down to atmospheric temperature before being filtered and concentrated *in vacuo*. The solid obtained was purified by flash chromatography (SiO_2, elution with 1% AcOH in 1:1 AcOEt/hexane) affording 2.23 g (75%) of desired γ-lactam **7b** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.89 (br. s, 1H), 4.77 (br. d, J= 11.5 Hz, 1H), 4.47 (dd, J= 11.5, 5.6 Hz, 1H), 4.08 (dd, J= 9.4, 5.0 Hz, 1H), 3.83 (s, 3H), 2.93 (m, 1H), 1.78-1.39 (m, 6H), 1.02-0.88 (m, 9H).

γ-Lactam 7a (R= Et)

By a procedure analogous to that described for preparing γ-lactam **7b**, aldol **5a** was hydrogenated at 55 p.s.i. for 48 h to provide γ-lactam **7a** in 72% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (br. s, 1H), 4.62 (br. d, J= 11.2 Hz, 1H), 4.51 (dd, J= 11.2, 5.4 Hz, 1H), 3.83 (s, 3H), 2.85 (m, 1H), 1.77-1.64 (m, 3H), 1.01 (t, J= 7.4 Hz, 3H), 0.98 (d, J= 6.9 Hz, 3H), 0.95 (d, J= 6.9 Hz, 3H).

γ-Lactam 7c (R= *n*-Bu)

A solution of aldol **5c** (361 mg, 0.80 mmol) in 6 mL 1:9 AcOH/MeOH, to which was added 250 mg 20% $Pd(OH)_2/C$, was vigorously shaken under 50 p.s.i. H_2 for 24 h. More catalyst (100 mg) was then added and the mixture was again shaken at 50 p.s.i. for another 24 h after which time it brought down to atmospheric temperature before being filtered. The filtrate was then heated to reflux for 30 min, cooled to room temperature and concentrated *in vacuo*. The solid obtained was co-evaporated once with toluene and purified by flash chromatography (SiO_2, elution with 4% MeOH/$CHCl_3$) affording 140 mg (61%) of desired γ-lactam **7c** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02 (br. s, 1H), 4.93 (br. d, *J*= 11.3 Hz, 1H), 4.46 (dd, *J*= 11.3, 5.5 Hz, 1H), 4.15-4.08 (m, 1H), 3.83 (s, 3H), 2.94-2.87 (m, 1H), 1.80-1.34 (m, 6H), 0.94 (d, *J*= 6.9 Hz, 3H), 0.89 (t, *J*= 7.2 Hz, 3H).

γ-Lactam 7d (R= *i*-Bu)

By a procedure analogous to that described for preparing γ-lactam **7c**, aldol **5d** was hydrogenated at 50 p.s.i. for 40 h and heated to reflux for 30 min providing γ-lactam **7d** in 61% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.92 (br. s, 1H), 4.81 (br. d, *J*= 11.5 Hz, 1H), 4.46 (m, 1H), 4.09 (m, 1H), 3.83 (s, 3H), 3.04-2.98 (m, 1H), 1.78-1.73 (m, 2H), 1.66-1.47 (m, 3H), 1.00-0.90 (m, 12H).

γ-Lactam 7e (R= CH_2Ph)

By a procedure analogous to that described for preparing γ-lactam **7c**, aldol **5e** was hydrogenated at 50 p.s.i. for 24 h and heated to reflux for 30 min providing γ-lactam **7e** in 71% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01 (br. s, 1H), 7.35-7.15 (m, 5H), 5.02 (br. d, *J*= 11.7 Hz, 1H), 4.40-4.34 (m, 1H), 4.06-4.01 (m, 1H), 3.84 (s, 3H), 3.34-3.27 (m, 1H), 3.10-3.04 (m, 2H), 1.84-1.72 (m, 1H), 0.98 (d, *J*= 6.7 Hz, 3H), 0.93 (d, *J*= 6.9 Hz, 3H).

β-Lactone 3b (R= *n*-Pr; 7-*n*-propyl-*clasto*-lactacystin β-lactone)

To a cold (0 °C) solution of γ-lactam **7b** (2.20 g, 8.06 mmol) in EtOH (100 mL) was added 0.1N aqueous NaOH (100 mL, 10.0 mmol). The mixture was stirred at room temperature for 15 h after which time H2O (50 mL) and AcOEt (100 mL) were added. The aqueous layer was then washed with AcOEt (2 x 50 mL), acidified with 6N aqueous HCl and concentrated *in vacuo* to a volume of *ca* 60 mL. This solution was then frozen and lyophilized. The obtained solid was suspended in THF, filtered to get rid of sodium chloride and concentrated *in vacuo* affording 2.05 g (98%) of the desired dihydroxyacid as white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 4.42 (d, *J*= 5.8 Hz, 1H), 3.90 (d, *J*= 6.5 Hz, 1H), 2.84 (m, 1H), 1.70-1.24 (m, 6H), 0.95-0.84 (m, 9H).

To a solution of the dihydroxyacid (1.90 g, 7.33 mmol) in anhydrous THF (36 mL) was added a solution of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 2.59, 8.06 mmol) in anhydrous MeCN (36 mL) followed by triethylamine (0.72 mL, 22.0 mmol). After stirring for 70 min at room temperature, some toluene was added and the mixture was concentrated *in vacuo* and co-evaporated 2 more times with toluene. Purification by flash chromatography (SiO_2, elution with 2:3 AcOEt/hexane) afforded 1.44 g (81%) of desired β-lactone **3b** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.07 (br. s, 1H), 5.26 (d, *J*= 6.1 Hz, 1H), 3.97 (dd, *J*= 6.4, 4.4 Hz, 1H), 2.70-2.63 (m, 1H), 2.03 (d, *J*= 6.4 Hz, 3H), 1.93-1.44 (m, 5H), 1.07 (d, *J*= 7.0 Hz, 3H), 0.99 (d, *J*= 7.3 Hz, 3H), 0.91 (d, *J*= 6.7 Hz, 3H).
LRMS (FAB) *m/e* 242 ($M+H^+$)

β-Lactone 3a (R= Et; 7-ethyl-*clasto*-lactacystin β-lactone)

Hydrolysis of **7a**, as described for **7b** above, afforded the corresponding dihydroxyacid in 100% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 4.45 (d, *J*= 5.8 Hz, 1H), 3.90 (d, *J*= 6.4 Hz, 1H), 2.74 (m, 1H), 1.71-1.53 (m, 3H), 0.94 (t, *J*= 7.4 Hz, 3H), 0.92 (d, *J*= 6.8 Hz, 3H), 0.88 (d, *J*= 6.8 Hz, 3H).

By a procedure analogous to that described for preparing β-lactone **3b**, β-lactone **3a** was obtained in 79% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.17 (br. s, 1H), 5.30 (d, *J*= 6.0 Hz, 1H), 3.98 (dd, *J*= 6.4, 4.4 Hz, 1H), 2.60 (m, 1H), 2.08 (d, *J*= 6.4 Hz, 3H), 1.97 (m, 2H), 1.75 (m, 1H), 1.12 (t, *J*= 7.5 Hz, 3H), 1.07 (d, *J*= 6.8 Hz, 3H), 0.92 (d, *J*= 6.8 Hz, 3H).

β-Lactone 3c (R= *n*-Bu; 7-*n*-butyl-*clasto*-lactacystin β-lactone)

Hydrolysis of **7c**, as described for **7b** above, afforded the corresponding dihydroxyacid in 100% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 4.42 (d, *J*= 5.8 Hz, 1H), 3.90 (d, *J*= 6.4 Hz, 1H), 2.86-2.79 (m, 1H), 1.70-1.24 (m, 8H), 0.97-0.86 (m, 9H).

By a procedure analogous to that described for preparing β-lactone **3b**, β-lactone **3c** was obtained in 40% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.14 (br. s, 1H), 5.27 (d, *J*= 6.1 Hz, 1H), 3.97 (d, *J*= 4.4 Hz, 1H), 2.68-2.61 (m, 1H), 1.94-1.86 (m, 2H), 1.72-1.36 (m, 7H), 1.07 (d, *J*= 7.0 Hz, 3H), 0.93 (t, *J*= 7.1 Hz, 3H), 0.91 (d, *J*= 6.8 Hz, 3H).
LRMS (FAB) *m/e* 256 ($M+H^+$)

β-Lactone 3d (R= *i*-Bu; 7-*i*-butyl-*clasto*-lactacystin β-lactone)

Hydrolysis of **7d**, as described for **7b** above, afforded the corresponding dihydroxyacid in 100% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 4.50 (d, *J*= 5.8 Hz, 1H), 4.00 (d, *J*= 6.5 Hz, 1H), 3.09-3.02 (m, 1H), 1.90-1.61 (m, 3H), 1.49-1.40 (m, 2H), 1.02 (d, *J*= 6.7 Hz, 3H), 0.98 (d, *J*= 6.5 Hz, 3H), 0.97 (d, *J*= 6.7 Hz, 3H).

By a procedure analogous to that described for preparing β-lactone **3b**, β-lactone **3d** was obtained in 62% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.16 (br. s, 1H), 5.25 (d, *J*= 6.1 Hz, 1H), 3.97 (d, *J*= 4.4 Hz, 1H), 2.71 (dd, *J*= 15.1, 6.2 Hz, 1H), 1.95-1.66 (m, 5H), 1.08 (d, *J*= 6.9 Hz, 3H), 0.99 (d, *J*= 6.3 Hz, 3H), 0.98 (d, *J*= 6.3 Hz, 3H), 0.92 (d, *J*= 6.7 Hz, 3H).

LRMS (FAB) *m/e* 256 ($M+H^+$)

β-Lactone 3e (R= CH_2Ph; 7-benzyl-*clasto*-lactacystin β-lactone)

Hydrolysis of **7e**, as described for **7b** above, afforded the corresponding dihydroxyacid in 88% yield. ^1H NMR (300 MHz, CD_3OD) δ 7.25-7.04 (m, 5H), 4.29 (d, *J*= 5.7 Hz, 1H), 3.83 (d, *J*= 6.4 Hz, 1H), 3.01-2.82 (m, 3H), 1.65 (m, 1H), 0.90 (d, *J*= 6.6 Hz, 3H), 0.86 (d, *J*= 6.8 Hz, 3H).

By a procedure analogous to that described for preparing β-lactone **3b**, β-lactone **3e** was obtained in 77% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 7.36-7.20 (m, 5H), 6.57 (br. s, 1H), 5.08 (d, *J*= 5.4 Hz, 1H), 3.94 (d, *J*= 4.5 Hz, 1H), 3.25 (d, *J*= 10.1 Hz, 1H), 3.01-2.89 (m, 2H), 1.92-1.81 (m, 1H), 1.05 (d, *J*= 6.9 Hz, 3H), 0.86 (d, *J*= 6.7 Hz, 3H). LRMS (FAB) *m/e* 290 ($M+H^+$)

Example 6: Pharmacokinetics of N-(Pyrazine)carbonyl-L-phenylalanine-L-leucine Boronic Acid* (1) *in Rats and Primates

A single dose intravenous pharmacokinetics study with *N*-(pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid (**1**) was conducted in Sprague-Dawley rats (140 to 280 g). Animals were assigned to 3 groups (6/sex in Groups 1 and 2; 9/sex in Group 3). Animals in groups 1, 2, and 3 received 0.03, 0.1 or 0.3 mg/kg of **1**, respectively, in the same dose volume.

Blood samples (approximately 1.0 mL) were collected from the jugular vein of animals pre-dose and at approximately 10 and 30 min and 1, 3 and 24 h post-dose on Day 1. The samples were assayed for **1** using a chromatography/mass spectroscopy (LC/MS/MS) method. The lower limit of quantitation for analysis was established at 2.5 ng/mL for **1** in rat plasma and whole blood.

Following the single intravenous doses, plasma or whole blood levels of **1** were only measurable at the 0.3 mg/kg dose level. The observed C_{max} occurred at the first time point; hence, the time to peak concentration (T_{max}) was estimated to be w 10 min in both male and female rats. Males generally had slightly higher peak concentration (C_{max}) and area under the concentration-time curve (AUC_{0-t}) values than females. The C_{max} values in plasma and in whole blood in males were 51.8 and 22.7 ng/mL, respectively and in females were 36.9 and 19.1 ng/mL,

respectively. The AUC_{0-t} values in plasma and whole blood in males were 14.0 and 18.6 ng•h/mL, respectively and in females were 12.9 and 17.7 ng•h/mL, respectively. Estimation of the elimination half-life ($t_{1/2}$) was not possible due to the fluctuation of **1** levels during the terminal phase. The observations suggest that **1** is rapidly cleared from the blood.

Example 7: Preparation of blood samples for in vitro measurement of 20S proteasome activity

This preparation procedure applies to blood samples collected from mammals, particularly human subjects, Cynomologus monkeys, rats and mice. Peripheral white blood cells are separated from blood samples upon collection for storage at about -70 °C until tested. To prevent interference with the fluroescence assay, it is important that the sample preparation remove all hemoglobin.

PROCEDURE

The required amount of blood is collected into a tube containing anticoagulant. For human subjects and primates, approximately 5 mL of blood is required; for rats, approximately 4 mL of blood is needed; for mice, approximately 1 mL of blood is needed from each of five mice, and the five blood samples are pooled to provide approximately 5 mL.

The blood sample is diluted 1:1 (v/v) with sterile saline, and the blood-saline mixture is layered over ~1 mL of Nycoprep™ separation medium (GIBCO BRL Products) in a 14 (75 mm polystyrene test tube. The sample is centrifuged at 500 (g for approximately 30 minutes at room temperature. The top layer is removed, leaving ~2-3 mm of the cell band between the top and bottom layers. The remaining cell band is transferred by pipette to a clean centrifuge tube. The cell band is washed with 3 mL of cold phosphate-buffered saline and centrifuged at 400 (g for 5 minutes at 4 °C. The supernatant is poured off and the pellet is resuspended in ~1 mL of cold phosphate-buffered saline. The suspension is transferred to a 1.5 mL Eppendorf microfuge tube and microfuged at 6600 (g for approximately 10 minutes at 4 °C. The

supernatant is aspirated off and the cell pellet is stored at -70 °C.

Example 8: Assay to measure 20S proteasome activity in peripheral white blood cells

The assay is based upon the SDS-inducible chymotrypsin-like activity of free 20S particles. It uses fluorometry to measure the rate at which the 20S proteasome hydrolyzes an amide bond in a small peptide substrate. Measurement of this rate in the absence and in the presence of an inhibitor allows a determination of how enzyme is bound by inhibitor. This assay is used to measure 20S proteasome activity in peripheral white blood cells in mammals, particularly humans, Cynomolgus monkeys, rats and mice.

ABBREVIATIONS:

AMC	7-amino-4-methylcoumarin
DMF	dimethyl formamide
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	disodium ethylenediaminetetraacetate
HEPES	N-(2-Hydroxyethyl)piperazine-N -(2-ethanesulfonic acid); pH adjustments with NaOH
Hgb	hemoglobin
SDS	sodium dodecylsulfate of either -
SDS-grade:	99% sodium dodecylsulfate
Lauryl grade:	~70% dodecyl sulfate with the remainder as tetradecyl and hexadecyl sulfates.
TMB	3,3,5,5 -tetramethylbenzidine
WBC	white blood cells
Ys substrate	N-Succinylleucylleucylvalyltyrosyl 7-amino-4-methylcoumarin (Suc-Leu-Leu-Val-Tyr-AMC)

PROCEDURE

White blood cells, prepared as described in Example 1, are lysed by adding 200 µL of 5 mM EDTA to each sample. The samples are allowed to stand on ice for at least 1 h. The Ys substrate is dissolved in DMSO. Purified 20S proteasome standard from rabbit reticulocytes is diluted 1:9 (v/v) in 20 mM HEPES/0.5 mM EDTA (pH 7.8). The Ys substrate buffer contains 20 mM HEPES, 0.5 mM EDTA, 0.35% SDS, and 60 µM Ys substrate.

Coomassie protein assay (measuring total protein content) and hemoglobin assay are performed on the test sample following standard procedures using commercially available kits. The amount of white blood cell protein present is calculated according to the following formula:

$$\text{WBC protein (µg/mL)} = \text{total protein (µg/mL)} - [\text{hemoglobin (µg/mL)} * 0.56]$$

The 20S proteasome standard is diluted 1:10 in 20 mM HEPES/0.5 mM EDTA (pH 7.8) to form a 12 µg/mL stock solution and placed on ice. 10 µL of the standard 20S proteasome solution is added to a cuvette and the reaction is run for 10 minutes. The maximum linear slope is measured on a fluorometer and provides a measurement of standard proteasome activity.

5 µL of a test sample is added to a cuvette containing 2 mL of Ys substrate buffer at 37 °C, and the reaction is allowed to run for 10 minutes. Complete activation of the 20S proteasome is achieved over 10 minutes. Consistent results are obtained for readings taken after 3 minutes and up to 10 minutes. The maximum linear slope for at least 1 minute of data is measured on a fluorometer. If the rate is less than 1 pmol AMC/sec, the measurement is repeated using 10 µL of the test sample.

The amount of 20S proteasome activity in the test sample is calculated according to the following formula:

$$\text{20S activity (pmol/s)} = \frac{\text{Rate (FU/min)} * \text{Ys calibration (pmol/FU)}}{0.0001 * \text{WBC protein (mg)} * 60 \text{ s/min}}$$

Example 9: Proteasome Activity Levels in Peripheral White Blood Cells of Human

Volunteers

Blood samples (approximately 2 mL each) were obtained on five occasions from five human volunteers over a period of ten weeks. After collection, white blood cells were isolated from the individual blood samples using a Nycoprep™. The resulting pellet was stored in a freezer set to maintain -60 °C to -80 °C until the day of testing. Samples collected on each occasion were tested together and each sample was tested in duplicate.

20S proteasome activity was determined by measuring the rate of proteolytic hydrolysis of a fluorescent (AMC)-tagged peptide substrate by the sample and normalizing the activity to the amount of cell-specific protein present in the lysate, as described in Example 2 above. 20S proteasome activity in the sample was determined from the equation:

$$\text{20S proteasome activity (pmol AMC/sec/mg protein)} = \frac{\text{(FU/sec) / (5 x } 10^{-6} \text{ mL)(protein } \mu\text{g/mL)}}{C}$$

where C = conversion factor equating the amount of fluorescence to the concentration of free AMC (FU/pmol AMC).

RESULTS AND DISCUSSION

Individual test data and average +/- standard error mean (SEM) for each sample are presented in Appendix A. The average 20S proteasome activity values found for each human volunteer ranged from 10.73 to 14.79 pmol AMC/sec/mg protein (Table 1 and Figure 1). The average 20S proteasome activity found in the population was 12.12 +/- 0.81 pmol AMC/sec/mg protein with individual observed values ranging from 53% to 165% of the average value.

Duplicate testing was performed on each sample. The variation in test duplicates (SEM%) on the five days of testing was 9.9%, 11.9%, 10.3%, 8.2% and 9.5%. For individual test duplicates this ranged from 0.8% to 22.9%. The average variation between test duplicates across the four days of testing was 10.0%. Further

test development to reduce variation is ongoing.

Table 1: 20S Proteasome Activity Levels in Human Volunteers

Volunteer	20S Proteasome Activity (pmol AMC/sec/mg protein)		Range as % of Average
	Average (SEM	Range	
A	14.79 (1.90	10.71 - 19.15	72.4 - 129.5%
B	12.02 (2.36	7.61 - 20.03	63.3 - 166.6%
C	11.19 (1.49	6.47 - 13.87	57.8 - 123.9%
D	11.86 (2.83	6.68 - 18.35	56.3 - 154.7%
E	10.73 (1.58	7.02 - 15.30	65.4 - 142.6%
Population Average	12.12 (0.81	6.47 - 20.03	53.4 - 165.3%

Table 2: Variation in Test Duplicates of 20S Proteasome Activity in Human Volunteers

Volunteer	Variation in Test Duplicates (SEM %)					
	10/15/97	11/3/97	11/13/97	12/2/97	12/22/97	Average Variation
A	0.8	8.9	13.6	11.7	5.3	
B	5.4	22.2	5.3	10.1	11.6	
C	10.5	20.1	10.1	5.1	12.4	
D	-	6.0	12.0	4.2	2.0	
E	22.9	2.1	-	9.9	16.0	
Average	9.9	11.9	10.3	8.2	9.5	10.0

Example 10: Temporal 20S Proteasome Activity in Isolated White Blood Cells and Tissues Following Administration of N-(Pyrazine)carbonyl-L-phenylalanine-L-leucine Boronic Acid (1)

GENERAL PROCEDURES

Dose formulations of **1** were prepared daily during the course of the study. Dilutions were prepared from a stock solution. The stock solution of **1** was made up in 98% saline (0.9%), 2% ethanol with 0.1% ascorbic acid. Dilutions of the stock were made in the same vehicle.

Female CD2-F1 mice (18 to 20 g), female BALB/c mice (18 to 20 g), female Wistar rats (150 to 200 g) and male Sprague-Dawley rats (250 to 450 g) were obtained from Taconic Farms (Germantown, NY). Animals were observed for at least one week and examined for general health before study initiation. Animals used in these studies were asymptomatic. Mice were housed 5 per cage and rats 3 per cage in polycarbonate cages. Corn Cob bedding (AND-1005; Farmers Exchange, Framingham, MA) was used during the observation and study periods. Fluorescent lighting was controlled to automatically provide alternate light and dark cycles of approximately 12 hours each. Temperature and humidity were centrally controlled and recorded daily and readings ranged between 21 +/- 2 °C and 45 +/- 5 °C, respectively. Pellets of standard rodent chow (#5001, Purina, St. Louis, MO) were available *ad libitum* throughout the observation and study periods. Cambridge city tap water was provided by water bottles *ad libitum*. No contaminants of food and water are known which would be expected to interfere with the study.

Drugs were administered in vehicle intravenously (IV) using a dose volume of 100 µL per mouse or 1.0 mL/kg in rats. Control groups were administered with the vehicle (98% saline [0.9%], 2% ethanol, 0.1% ascorbic acid). Animals were dosed with **1** as a single bolus given either once or on multiple occasions. Animals exhibiting moribund activity were euthanized with CO_2 inhalation.

Following IV dosing with **1**, blood was withdrawn at various time points and peripheral white blood cells were isolated. The tissues collected were brain, colon, liver, muscle (gastrocnemius), prostate and testes.

Ex Vivo 20S Proteasome Activity Determined in Peripheral White Blood Cells of Mice After Single Intravenous Administration of 1

In two combined studies, female CD2-F1 mice (18 to 20 g) and female BALB/c mice (18 to 20 g) were administered a single intravenous dose of **1** (0.1 to 3.0 mg/kg in a dose volume of 100

μL). The vehicle was 98% saline [0.9%], 2% ethanol, 0.1% ascorbic acid. Blood samples were collected at 1.0 and 24 h following administration. Due to the blood volume required in the 20S proteasome activity assay, groups of five mice were sacrificed at the same time and their blood pooled to generate single data points.

There was a significant ($p < 0.05$) dose-related decrease in 20S proteasome activity for all dose groups at 1.0 h following intravenous administration of **1** (Figure 1) which starts to recover at 24 h (Figure 2). These studies demonstrated a dose-dependent and reversible inhibition of 20S proteasome activity in the peripheral white blood cells of mice following administration of a single intravenous injection of **1**.

Ex Vivo 20S Proteasome Activity Determined in Peripheral White Blood Cells of Rats After Single Intravenous Administration of 1

In four combined studies, female Wistar rats (150 to 200 g) were administered a single intravenous dose of **1** (0.03 to 0.3 mg/kg in a dose volume of 1.0 mL/kg). The vehicle was 0.1% ascorbic acid/2% ethanol/98% saline (0.9%). Blood samples were collected at 1.0, 24 and 48 h following administration of **1**.

There was a significant ($p < 0.05$) dose-related decrease in 20S proteasome activity at 1.0 h following intravenous administration of **1** (Figure 3). Twenty-four hours after administration, the dose-related decreases in 20S proteasome activity were smaller, but remained significant ($p < 0.05$) in the higher dose groups, (0.2 mg/kg (Figure 4). At 48 h after administration, 20S proteasome activity was no longer significantly decreased (Figure 5).

These studies demonstrated a dose-dependent and reversible inhibition of 20S proteasome activity in the peripheral white blood cells of rats following administration of a single intravenous injection of **1**. A slower rate of return to baseline for 20S proteasome activity levels was observed in rats, possibly indicating faster metabolism of **1** in mice.

Ex Vivo 20S Proteasome Activity Determined in Peripheral White Blood Cells of Rats After Repeat Intravenous Administration of 1

When daily intravenous **1** was administered for 7 days, a dose-related decrease in 20S proteasome activity was observed 24 h after administration of the last dose. Significant inhibition was observed for doses 0.05 mg/kg. The extent of 20S proteasome inhibition observed 24 h after administration of 7 daily intravenous doses was greater than that observed 24 h after administration of a single intravenous dose and probably reflects a cumulative effect of daily administration of **1** on its biological target, the proteasome.

A significant dose-related decrease in 20S proteasome activity was observed 24 h after

administration of the last dose for alternate daily intravenous administration of **1** for 14 days. The dose-related decreases in 20S proteasome activity were significant ($p < 0.05$) in the dose groups 0.2 mg/kg. A significant ($p < 0.05$) dose-related decrease in 20S proteasome activity was also observed 24 h after administration of the last dose for once weekly intravenous administration of **1** for 8 weeks. The dose-related decreases in 20S proteasome activity were significant ($p < 0.05$) in the dose groups > 0.1 mg/kg.

In a fourth repeat dose study, male Sprague-Dawley rats (250 to 450 g; n = 6 per group) were treated with twice weekly intravenous doses of **1** (0.01 to 0.35 mg/kg/day in a dose volume of 1.0 mL/kg) for two weeks. The vehicle was 0.1% ascorbic acid/2% ethanol/98% saline (0.9%). Blood samples were collected 1.0 h after the last dose for evaluation of 20S proteasome activity.

When **1** was administered twice weekly for 2 weeks (total of 4 doses), a dose-related decrease in 20S proteasome activity was observed 1.0 h after the last dose (Figure 6). The dose-related decreases in 20S proteasome activity were significant ($p < 0.05$) for all dose groups 0.03 mg/kg.

The results indicate that repeat dose administration of **1** elicits a dose-related decrease in 20S proteasome activity in rat white blood cells. The extent of inhibition of 20S proteasome activity is greater than that seen after a single dose when **1** is given daily or every other day. When the interval between doses of **1** is increased to allow for recovery (i.e., once weekly regimens), the degree of inhibition is equivalent to single administration of **1**. This pharmacodynamic profile supports twice weekly dosing with **1**, wherein transient inhibition is observed.

Ex Vivo 20S Proteasome Activity Determined in Rat Tissues After Repeat Intravenous Administration of 1

In two studies, female Wistar rats (150 to 200 g) were administered a single intravenous dose of **1** (0.03, 0.1 and 0.3 mg/kg in a dose volume of 1.0 mL/kg). The vehicle was 0.1% ascorbic acid/2% ethanol/98% saline (0.9%). Tissue samples were collected from liver and brain at 1.0, 24 and 48 h following administration for evaluation of 20S proteasome activity.

There was a significant ($p < 0.05$) dose-related decrease in 20S proteasome activity in rat liver at 1.0 h following intravenous administration of **1**. Twenty-four hours after administration, the dose-related decreases in 20S proteasome activity were smaller, but remained significant ($p < 0.05$) in the high dose group, 0.3 mg/kg. At 48 h after administration, the 20S proteasome activity in rat liver had returned to baseline. The extent of 20S proteasome inhibition in the liver returned to baseline levels faster than that observed for peripheral white blood cells. No 20S proteasome inhibition was observed in brain tissue, reflecting the lack of penetration of **1** into this tissue.

In a third study, male Sprague-Dawley rats (250 to 450 g) were administered a single intravenous dose of **1** (0.1 and 0.3 mg/kg in a dose volume of 1.0 mL/kg). The vehicle was 0.1%

ascorbic acid/2% ethanol/98% saline (0.9%). Blood and tissue samples were collected 1.0 h following administration for evaluation of 20S proteasome activity. The tissues collected were brain, colon, liver, muscle (gastrocnemius), prostate and testes.

Significant ($p < 0.05$) dose-related decreases in 20S proteasome activity were observed in peripheral white blood cells, colon, liver, muscle (gastrocnemius), and prostate at 1.0 h following intravenous administration of **1**. No 20S proteasome inhibition was observed in brain and testes, reflecting the lack of **1** penetration into these tissues.

The 20S proteasome inhibition in tissues 1.0 h after intravenous dose administration, except for brain and testes, was similar to that observed for peripheral white blood cells.

Ex Vivo 20S Proteasome Activity Determined in Primates After Single Intravenous Administration of 1

Male and female Cynomolgus monkeys (2.2 to 3.5 kg) were assigned to four groups (5/sex/group). Each group received 0 (vehicle control), 0.045, 0.067 or 0.100 mg/kg/dose of **1** as a single intravenous injection in a dose volume of 0.3 mL/kg twice weekly for 4 weeks (days 1, 5, 8, 12, 15, 19, 22 and 26). The vehicle was 0.1% ascorbic acid/2% ethanol/98% saline (0.9%). Three males from the control, low- and mid-dose groups, two high-dose males, and three females/group were sacrificed at the end of treatment on Day 27. Two animals/sex/group were designated as recovery animals and received treatment for 4 weeks followed by 2 weeks of recovery; they were sacrificed on Day 41.

Blood was collected for 20S proteasome activity determination prior to treatment, at 1.0 h after dosing on Days 1, 8, 15 and 22, and at 1.0 h prior to dosing on Days 5, 12, 19 and 26; and on Days 31, 34, 38, and 41 (recovery sacrifice animals). Blood was also collected for 20S proteasome activity determination from the high-dose male before it was sacrificed in moribund condition on Day 26 after receiving 8 doses.

Determination of white blood cell 20S proteasome activity 1.0 h after dosing revealed a significant and dose-related decrease in enzyme activity that recovered prior to the subsequent dose (Figures 7 and 8). The moribund animal was found to have low residual 20S proteasome activity in its white blood cells at sacrifice on Day 26.

These data support a twice weekly treatment regimen for **1**, since the 20S proteasome levels recover between doses.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the

invention and appended claims.

What is claimed is:

1. A method for treating a patient afflicted with multiple sclerosis comprising administering to the patient an effective amount of an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof.

2. The method according to claim 1, wherein the agent is administered in an amount sufficient to reduce the frequency or severity of relapse.

3. The method according to claim 1, wherein the agent is a proteasome inhibitor.

4. The method according to claim 3, wherein the proteasome inhibitor is lactacystin or a lactacystin analog compound.

5. The method according to claim 4, wherein the lactacystin analog compound is selected from the group consisting of lactacystin, *clasto*-lactacystin β-lactone, 7-ethyl-*clasto*-lactacystin β-lactone, 7-*n*-propyl-*clasto*-lactacystin β-lactone, and 7-*n*-butyl-*clasto*-lactacystin β-lactone.

6. The method according to claim 5, wherein the lactacystin analog compound is 7-*n*-propyl-*clasto*-lactacystin β-lactone.

7. A method for treating a patient afflicted with asthma comprising administering to the patient an effective amount of an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof.

8. The method according to claim 7, wherein the agent is administered in an amount

sufficient to reduce the frequency or severity of asthmatic attack.

9. The method according to claim 7, wherein the agent is a proteasome inhibitor.

10. The method according to claim 9, wherein the proteasome inhibitor is lactacystin or a lactacystin analog compound.

11. The method according to claim 10, wherein the lactacystin analog compound is selected from the group consisting of lactacystin, *clasto*-lactacystin β-lactone, 7-ethyl-*clasto*-lactacystin β-lactone, 7-*n*-propyl-*clasto*-lactacystin β-lactone, and 7-*n*-butyl-*clasto*-lactacystin β-lactone.

12. The method according to claim 11, wherein the lactacystin analog compound is 7-*n*-propyl-*clasto*-lactacystin β-lactone.

13. A method for treating a patient afflicted with asthma comprising administering to the patient an effective combination of a glucocorticoid and an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof.

14. The method according to claim 13, wherein the combination is administered in an amount sufficient to reduce the frequency or severity of asthmatic attack.

15. The method according to claim 13, wherein the glucocorticoid and the agent are administered at the same time.

16. The method according to claim 13, wherein the glucocorticoid and the agent are administered at different times.

17. The method according to claim 13, wherein the combination comprises an amount of the glucocorticoid that is less than its standard recommended dosage.

18. The method according to claim 13, wherein the combination comprises an amount of the agent sufficient to reduce the dose or treatment frequency required for the glucocorticoid.

19. The method according to claim 13, wherein the combination comprises an amount of the glucocorticoid sufficient to reduce the dose or treatment frequency required for the agent.

20. The method according to claim 13, wherein the agent is a proteasome inhibitor.

21. The method according to claim 20, wherein the proteasome inhibitor is lactacystin or a lactacystin analog compound.

22. The method according to claim 21, wherein the lactacystin analog compound is selected from the group consisting of lactacystin, *clasto*-lactacystin β-lactone, 7-ethyl-*clasto*-lactacystin β-lactone, 7-*n*-propyl-*clasto*-lactacystin β-lactone, and 7-*n*-butyl-*clasto*-lactacystin β-lactone.

23. The method according to claim 22, wherein the lactacystin analog compound is 7-*n*-propyl-*clasto*-lactacystin β-lactone.

24. The method according to claim 13, wherein the glucocorticoid is selected from the group consisting of flunisolide, triamcinolone acetonide, beclomethasone dipropionate, dexamethasone sodium phosphate, fluticasone propionate, budesonide, hydrocortisone, prednisone, prednisolone, mometasone, tipredane, and butixicort.

25. The method according to claim 24, wherein the glucocorticoid is budesonide.

26. The method according to claim 13, wherein the agent is 7-*n*-propyl-*clasto*-lactacystin β-lactone and the glucocorticoid is budesonide.

27. A pharmaceutical composition comprising an effective combination of a glucocorticoid and an agent selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-κB via the ubiquitin proteasome pathway, and mixtures thereof.

28. The composition of claim 27, wherein said composition is provided in a unit dosage form.

29. The composition of claim 28, wherein the unit dosage form comprises an amount of the glucocorticoid that is less than its standard recommended dosage.

30. The composition of claim 27, wherein said composition comprises the agent in an amount sufficient to reduce the dose or treatment frequency required for the glucocorticoid.

31. The composition of claim 27, wherein the agent is a proteasome inhibitor.

32. The composition of claim 31, wherein the proteasome inhibitor is lactacystin or a lactacystin analog compound.

33. The composition of claim 32, wherein the lactacystin analog compound is selected from the group consisting of lactacystin, *clasto*-lactacystin β-lactone, 7-ethyl-*clasto*-lactacystin β-lactone, 7-*n*-propyl-*clasto*-lactacystin β-lactone, and 7-*n*-butyl-*clasto*-lactacystin β-lactone.

34. The composition according to claim 33, wherein the lactacystin analog compound is 7-*n*-propyl-*clasto*-lactacystin β-lactone.

35. The composition according to claim 27, wherein the glucocorticoid is selected from the group consisting of flunisolide, triamcinolone acetonide, beclomethasone dipropionate, dexamethasone sodium phosphate, fluticasone propionate, budesonide, hydrocortisone, prednisone, prednisolone, mometasone, tipredane, and butixicort.

36. The composition according to claim 35, wherein the glucocorticoid is budesonide.

37. The composition according to claim 27, wherein the agent is 7-*n*-propyl-*clasto*-lactacystin β-lactone and the glucocorticoid is budesonide.

Figure 1

Effect of 3b treatment on R-EAE

Day 22 through day 40

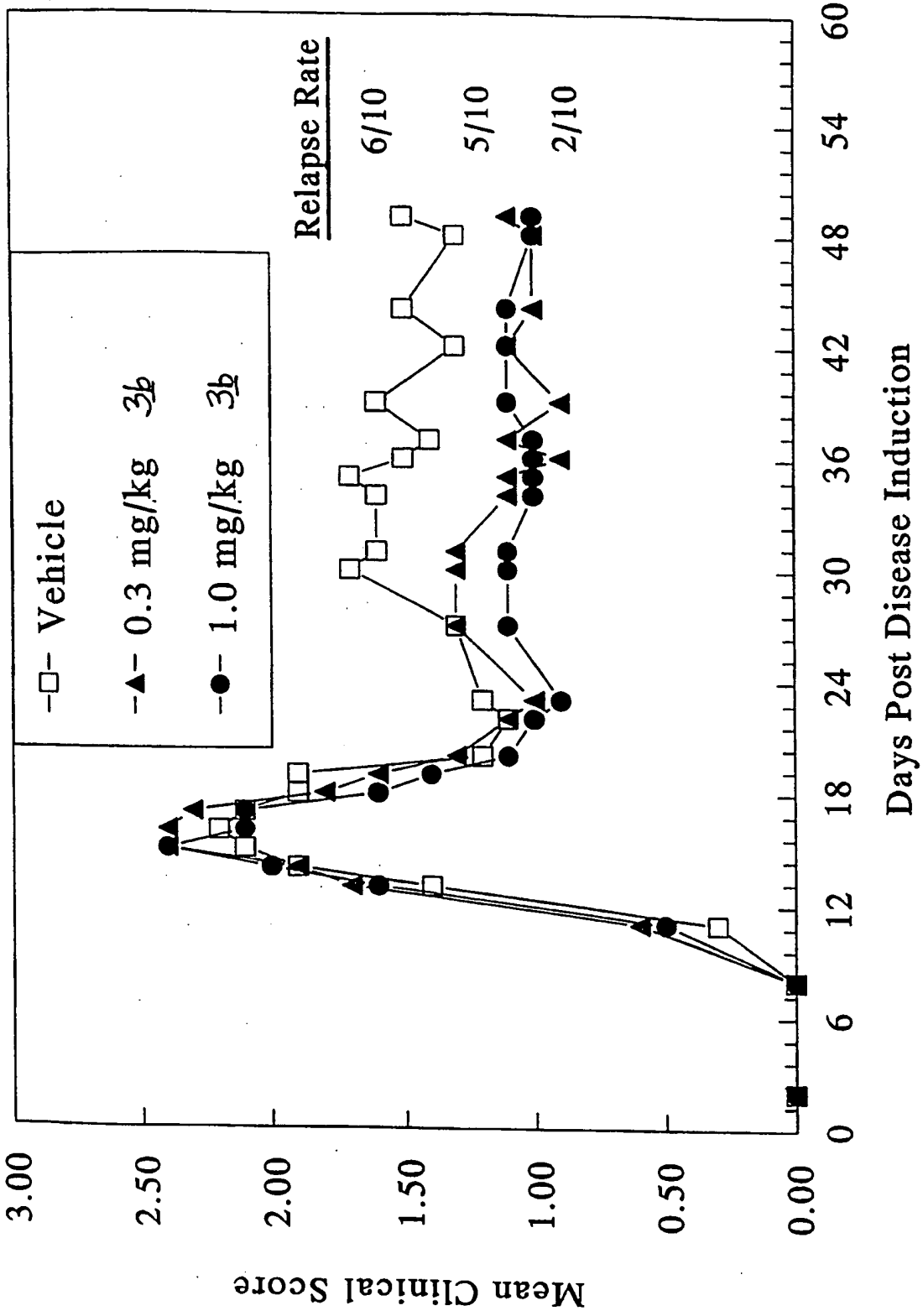
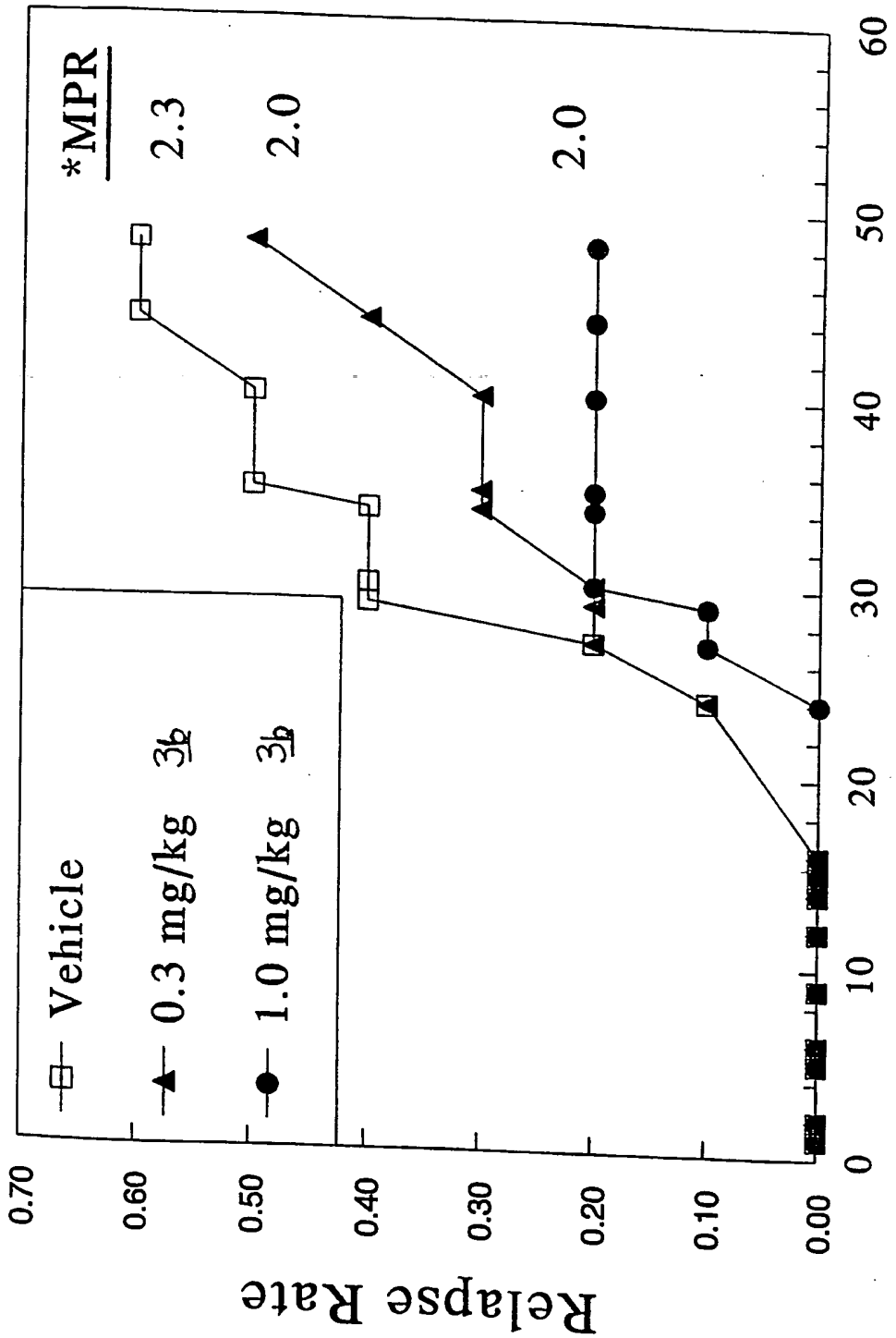


Figure 2 Effect of [illegible] treatment on Relapse



*Mean Peak of Relapse

Figure 3

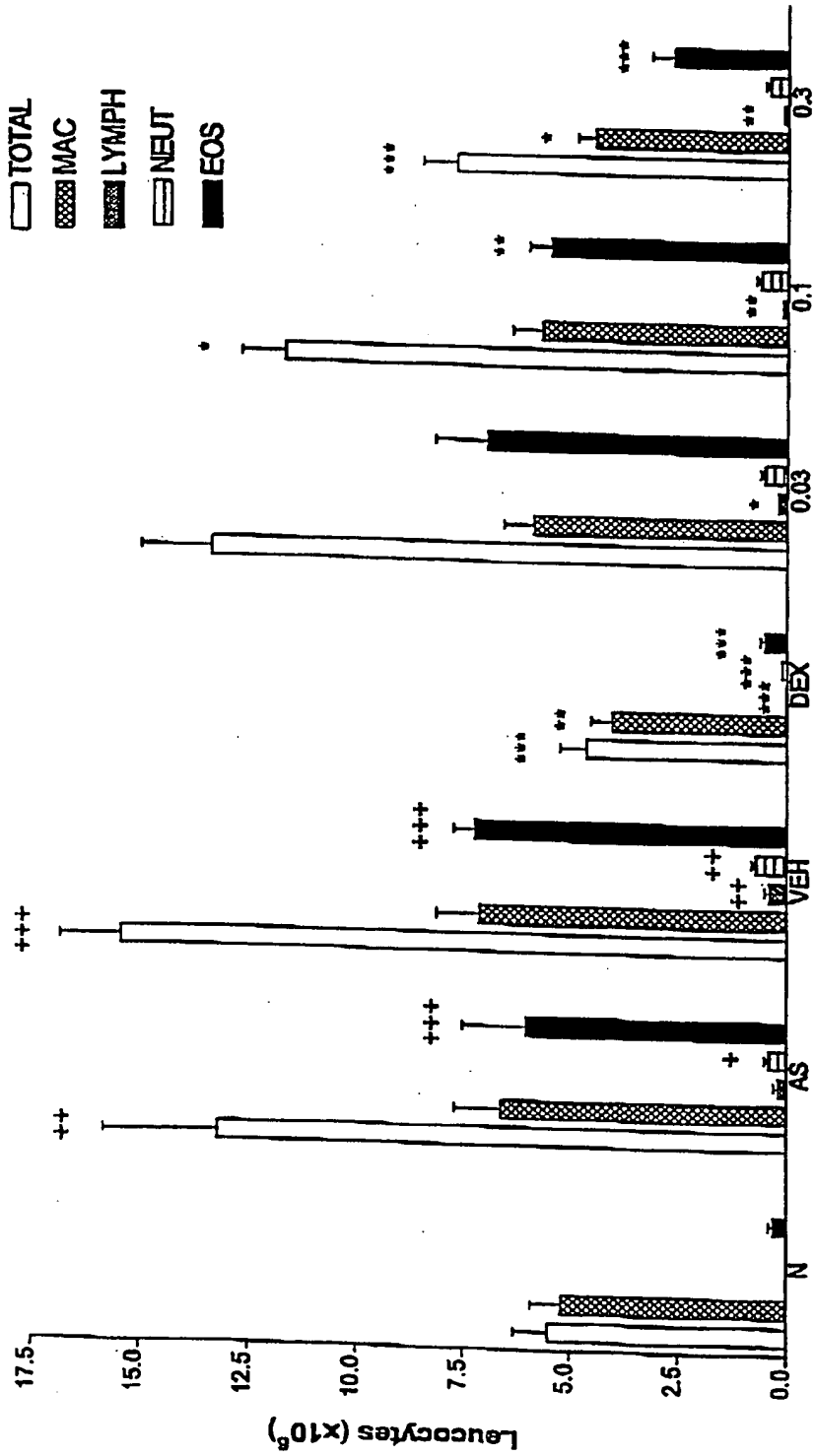


Figure 1. Leucocytes (mean ± s.e. mean) in bronchoalveolar lavage fluid from naive (N) or actively sensitized (AS) Brown Norway rats 72 h following exposure to aerosolized ovalbumin (10 mg/ml). Rats were treated 1 h prior to and 24 h and 48 h following challenge with vehicle (VEH; lactose 1 mg i.t.) dexamethasone (DEX; 0.1 mg/kg i.t.) or 3b (0.03, 0.1 or 0.3 mg/kg i.t.) + $P<0.05$, ++ $P<0.02$, +++ $P<0.001$ vs naive, * $P<0.05$, ** $P<0.02$, *** $P<0.001$ vs vehicle

Figure 4

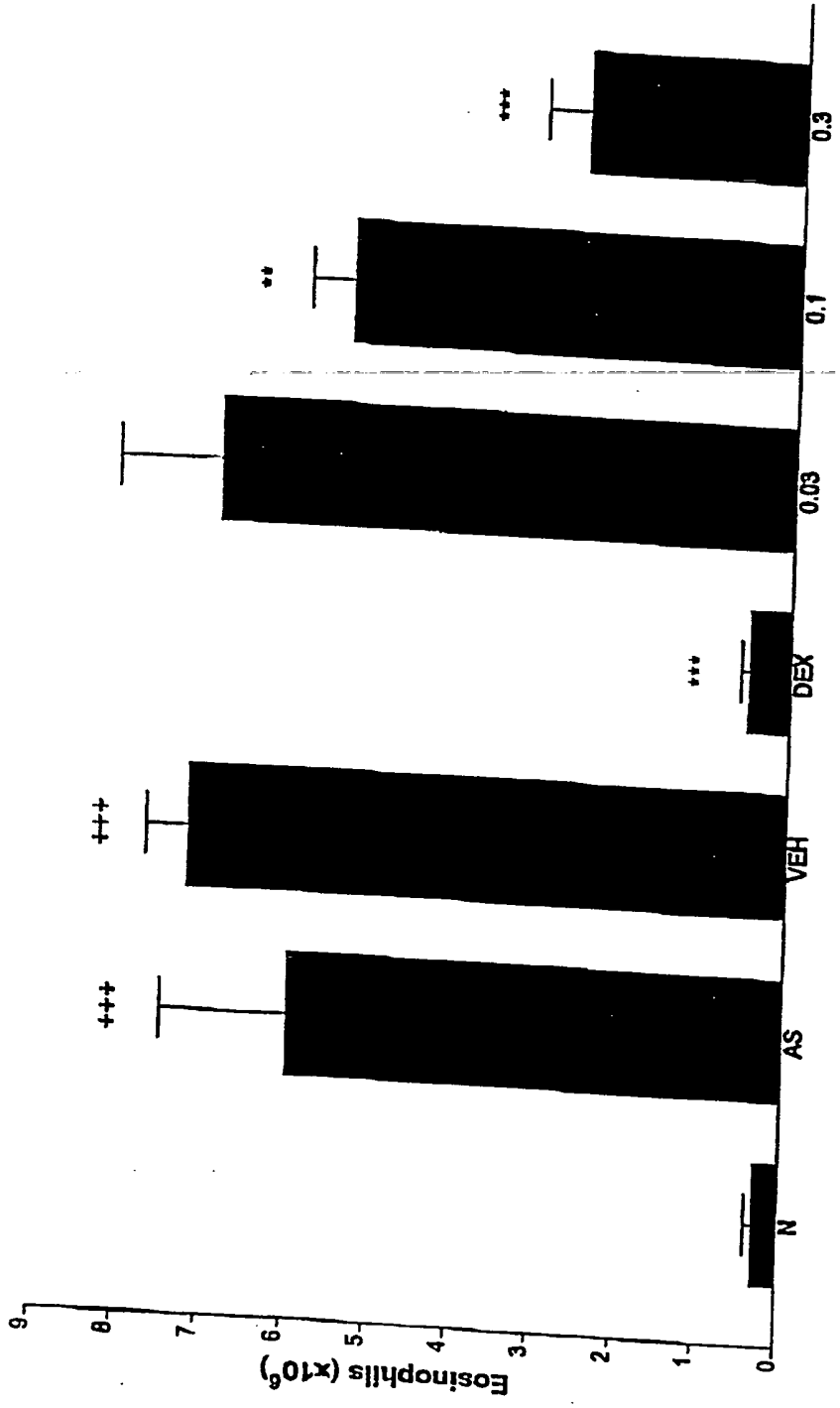
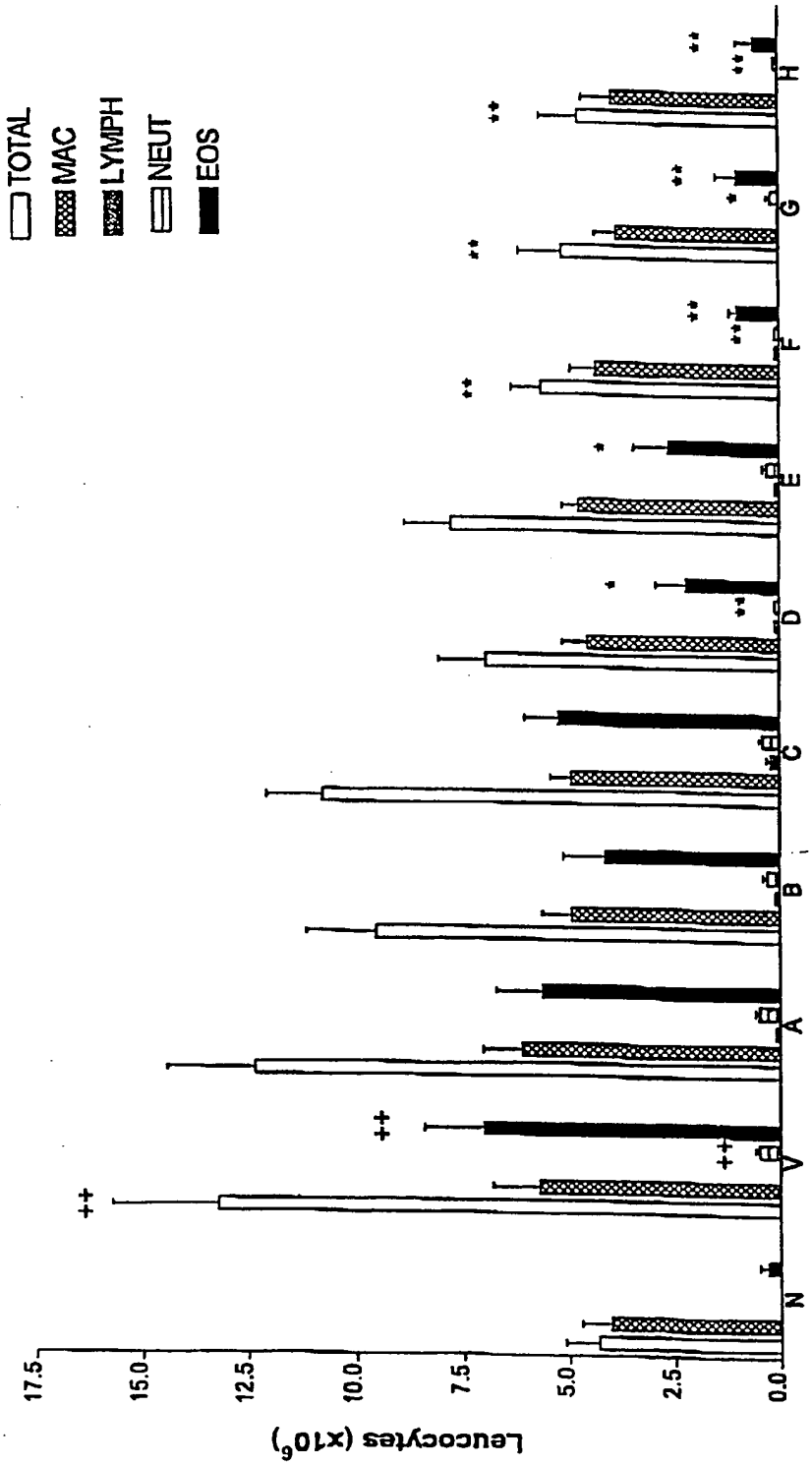


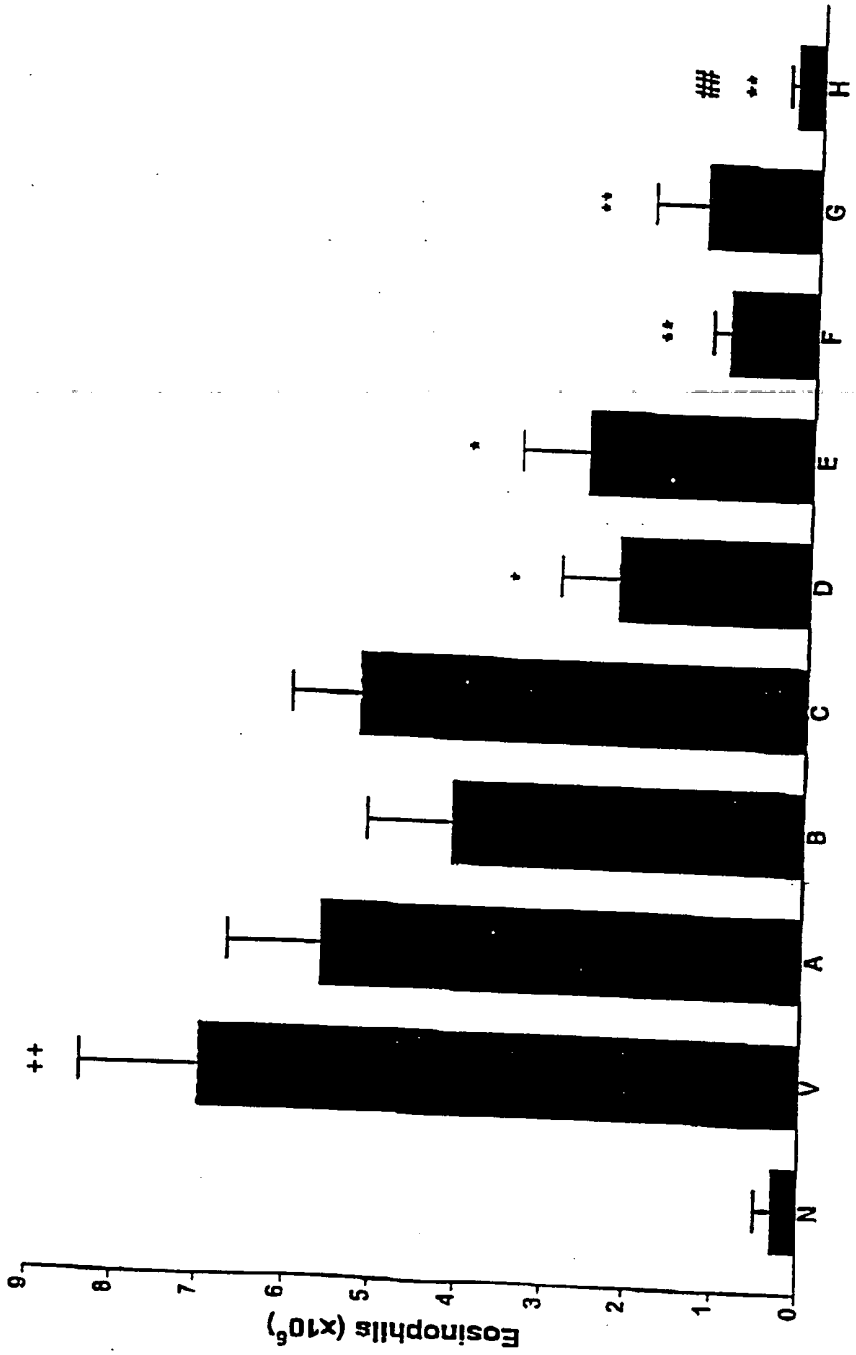
Figure 2. Eosinophils (mean ± s.e. mean) in bronchoalveolar lavage fluid from naive (N) or actively sensitized (AS) Brown Norway rats 72 h following exposure to aerosolized ovalbumin (10 mg/ml). Rats were treated 1 h prior to and 24 h and 48 h following challenge with vehicle (VEH; lactose 1 mg i.t.) dexamethasone (DEX; 0.1 mg/kg i.t.) or 3b (0.03, 0.1 or 0.3 mg/kg i.t.) +++ $P<0.001$ vs naive, ** $P<0.02$, *** $P<0.001$ vs vehicle

Figure 5



Leucocytes (mean ± s.e. mean) in bronchoalveolar lavage fluid from naive (N) or actively sensitized Brown Norway rats 72 h following exposure to aerosolized ovalbumin (10 mg/ml). Rats were treated 1 h prior to and 24 h and 48 h following challenge with vehicle (V; lactose, 1 mg i.t.), budesonide (C; 0.1 or F; 0.5 mg/kg i.t.), 3b (A; 0.03 or B; 0.1 mg/kg i.t.) or mixtures of budesonide and 3b (D; 0.1/0.03, E; 0.1/0.1, G; 0.5/0.03, H; 0.5/0.1 mg/kg i.t. respectively). ++ $P<0.02$ vs naive; * $P<0.05$, ** $P<0.02$ vs vehicle.

Figure 6



Eosinophils (mean ± s.e. mean) in bronchoalveolar lavage fluid from naive (N) or actively sensitized Brown Norway rats 72 h following exposure to aerosolized ovalbumin (10 mg/ml). Rats were treated 1 h prior to and 24 h and 48 h following challenge with vehicle (V; lactose, 1 mg i.t.), budesonide (C; 0.1 or F; 0.5 mg/kg i.t.), 3b (A; 0.03 or B; 0.1 mg/kg i.t.) or mixtures of budesonide and 3b (D; 0.1/0.03, E; 0.1/0.1, G; 0.5/0.03, H; 0.5/0.1 mg/kg i.t. respectively). ++ $P<0.02$ vs naive; * $P<0.05$, ** $P<0.02$ vs vehicle, ## $P<0.02$ vs budesonide (0.5 mg/kg i.t.).

INTERNATIONAL SEARCH REPORT

Ir. ational Application No
PCT/US 98/20065

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/69 A61K31/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 32105 A (HARVARD COLLEGE ;SCHREIBER STUART L (US); STANDAERT ROBERT F (US);) 17 October 1996	1-11
Y	see page 81, line 1 - line 17 see page 90, line 16 - line 18 see page 90, line 23 - line 25 see page 90, line 27 see examples 5-9 see claim 23, compounds: J6, J12, J13, J15, P2, P3, P4, P5	13-37
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 December 1998	12/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Trifilieff-Riolo, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir. ational Application No
PCT/US 98/20065

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X P,Y	US 5 780 454 A (GRENIER LOUIS ET AL) 14 July 1998 see column 7, line 5 - line 9 see column 26, line 44 - line 47 see column 47 - column 55; table II	7-9 13-20, 24,25, 27-31, 35,36
Y	"Martindale 13th edition" 1993 , ROYAL PHARMACEUTICAL SOCIETY , LONDON XP002088071 see page 722, left-hand column: asthma	13-37
Y	"the Merck Index 12th edition" 1996 , MERCK RES. LAB. , WHITEHOUSE STATION NJ XP002088072 see page 240 section 1490	13-37
Y	MANNING ET AL: "transcription inhibitors in inflammation" EXPERT OPINION ON INVESTIGATIONAL DRUGS, vol. 6, no. 5, May 1997, pages 555-567, XP002088067 see page 561 - page 562, paragraph 1	7-37
Y	LEVAN ET AL: "glucocorticoid receptor signaling in a bronchial epithelial cell line" AM. J. OF PHYSIOLOGY, vol. 16, May 1997, pages L838-L843, XP002088068 see abstract	7-37
Y	DEVILLIER ET AL: "action anti-inflammatoire des glucocorticoides" REVUE FRANÇAISE D'ALLERGOLOGIE ET D'IMMUNOLOGIE CLINIQUE, vol. 36, 1996, pages 937-961, XP002088069 see abstract see page 940, right-hand column, paragraph 2 - paragraph 3	7-37
Y	STACEY ET AL: "the allergen der P1 induces NF-KB activation through interference with IkBalpha fucntion in asthmatic bronchial epithelial cells" BIOCHEM. AND BIOPHYS. RES. COMM., vol. 236, no. 2, 1997, pages 522-526, XP002088070 see abstract see page 525, left-hand column, paragraph 3 - right-hand column, paragraph 1	7-37

3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/20065

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-26
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
In view of the large number of compounds which are defined by the wording of the claims, the search has been performed on the general idea and compounds mentioned in the examples of the description.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/US 98/20065

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9632105 A	17-10-1996	AU	5542396 A	30-10-1996
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